University of Alberta

Stabilization of Oil/water Emulsions by Hydrophobic Bacteria

by

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ABSTRACT

Bacterial degradation of hydrocarbons is a major route for removal of petroleum pollutants from the environment. This process requires interaction between the hydrated microbial surface and hydrophobic hydrocarbons. Even though mechanisms to facilitate contact between hydrocarbons and bacterial surfaces are known, including direct bacterial attachment to the oil/water interface and release of extracellular compounds, the actual mechanism involved in hydrocarbon transport is a debatable issue.

The aim of this study was to determine the relationship between bacterial surface properties, bacterial adhesion to oil/water interfaces and stabilization of emulsions.

It was found that intact bacterial cells were able to stabilize emulsions without changing the interfacial tension, by inhibition of droplet coalescence similar to emulsion stabilization by silica particles. Contact angle measurements revealed values in the range of 40° to 123° and were in qualitative agreement with the stabilized emulsion volumes, which ranged up to 80% of the mixture total volume.

In memory of

my mother Georgeta and my grandmother Victoria

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I INTRODUCTION

Bioremediation is considered the most environmentally sound and least intrusive of the promising new technologies in the field of remediation (Baker and Herson, 1994). The expansion of petroleum development and the spillages which occur during routine operations and as a consequence of acute accidents has resulted in hydrocarbon-contamination becoming a major environmental problem. These hydrocarbon pollutants have contaminated marine, fresh-water, and soil ecosystems, threatening human health and damaging the environment (Atlas and Cerniglia, 1995). Bioremediation offers several advantages over conventional cleanup technologies for surface, ground water and soil because it can be faster, safer and less costly.

Microbial cells play a very important role not only in bioremediation of hydrocarbon pollutants but also in bioprocesses in which petroleum hydrocarbons are converted into valuable products, for example α, ω dicarboxylic acids, which are difficult to obtain using conventional methods.

However, the chemical heterogeneity and water insolubility of crude oil define the problem and present a challenge to microorganisms using petroleum as a growth substrate. Enhancing the bioavailability of an insoluble substrate is dependent on understanding the mechanisms by which microbes transport and uptake insoluble substrates. At least two possible pathways for making hydrocarbons available to the microorganisms are known: direct contact (adherence) between microorganisms and oil droplets and release of extracellular surfactants or emulsifiers to increase the hydrocarbon-aqueous interfacial area.

In order to investigate the hydrocarbon transport processes into microbial cells, it is important to study the properties of the microbial cell surface and the behavior

and action of microorganisms towards hydrocarbon droplets. The study of microbial cell surface hydrophobicity is relevant in explaining microbial adhesion to insoluble substrates and stabilization of oil/water emulsions.

The hypothesis underlying this study was that bacterial cells could stabilize oil-water emulsions by steric hindrance against coalescence. The same mechanism was observed with fine divided particles, possessing different degrees of hydrophobicity, in stabilization of oil-water emulsions. First, the relationship between the surface properties of different bacterial strains and their adhesion to oil/water interfaces and stabilization of emulsions of hexadecane and water was investigated. The experiments were conducted with four bacterial species. The contact angles of the cells with water and hexadecane were measured and the interfacial tension of oil droplets with adherent bacteria was determined by the micropipette technique. Then, the results obtained during the experiments performed with bacterial cells were compared to results available for fine particles.

II LITERATURE SURVEY

2.1 ROLE OF MICROORGANISMS IN HYDROCARBON DEGRADATION

There are innumerable strains of microorganisms under the basic categories of bacteria and fungi, which are able to transform or degrade petroleum hydrocarbons into simpler, more desirable products as in bioprocesses (Singer and Finnerty, 1984), or less harmful and toxic substances as in bioremediation (Leahy and Colwell, 1990). The use of microbes in these processes takes advantages of the fact that microorganisms, like all living organisms, need carbon, energy and nutrients (such as nitrogen, phosphorus and trace metals) to survive. In recent years, the focus on using microorganisms in removal of oil spills and transformation of hydrocarbons into more valuable products has considerably increased. Bioremediation and bioprocesses are becoming a promising alternative to conventional methods such as incineration and oil scavenging because they can be safer, less costly and more efficient (Atlas and Cerniglia, 1995).

2.1.1 Bioremediation of Petroleum Hydrocarbons

The foundation of bioremediation has been the observation that over long periods of time and without human intervention, nature eliminates both natural and most man made pollution through natural processes (Baker and Herson, 1994). Petroleum entering an ecosystem provides a source of carbon and energy for microbial growth resulting in cell biomass and carbon dioxide that can be readily accommodated in

the environment (Atlas and Pramer, 1990, Alexander, 1999). Microorganisms carry out biodegradation in many different types of environments; of particular relevance for pollutants or potential pollutants are soils, groundwater, surface waters, and oceans (National Academy of Science, 1985; Rosenberg *et al.* 1992). By far, the largest and best-documented use of bioremediation to date was the cleaning of the Exxon Valdez spill in Prince William Sound, Alaska (Pritchard *et al.*, 1992). The spillage of more than 200,000 barrels of crude oil has brought attention to the role of microbial hydrocarbon degradation in the removal of petroleum pollutants. Even though the initial approach to the cleanup of the oil spilled from Exxon Valdez was physical, it turned out to be expensive and not efficient; therefore, bioremediation was considered as an alternative method. Bioremediation treatment restored the rocks to their original whiteness, which provided a contrast to the black oily untreated shorelines and gave visual credence to its effectiveness (Bragg *et al.*, 1994; Atlas and Cerniglia, 1995). The application of hydrocarbon microbiology to solve pollution problems in water and soil has an enormous economic and environmental importance (Prince, 1993).

2.1.1.1 Bioavailability of Hydrophobic Substrate

Petroleum is a naturally occurring, highly complex mixture of organic compounds containing homologous series, including alkanes, isoalkanes, alkyl aromatic and heteroatomic compounds (Eastcott *et al.*, 1988). A majority of these molecules is indeed composed solely of carbon and hydrogen, but most oils contain a small percentage of organic sulfur, organic nitrogen, and trace amounts of metallic constituents. As a major component of petroleum, alkanes have become an important source of pollution in the environment because of their extensive use (Walter *et al.*,

1997). The low solubility of alkanes in water causes them to separate out and form a two-phase system (Hommel, 1990). Utilization of alkanes by microorganisms poses the problem of contact between the two immiscible substrates and the microbial cell. Therefore, the major problem to be overcome by microorganisms in hydrocarbon degradation is to make the hydrophobic carbon source accessible to the cells (Rosenberg *et al.*, 1998).

2.1.1.2 Sources of Hydrocarbon Pollution

The manufacture, transportation and distribution of petroleum during the last century has resulted in hydrocarbon contamination becoming a major environmental problem (Atlas and Cerniglia, 1995; Atlas, 1981). The polluting sources include natural seeps, spillage during petroleum production and transportation, refinery wastewaters, spillage associated with plane, auto, and boat transportation, condensation from exhausts of gasoline and diesel engines, runoff from roads and fueling areas (Atlas, 1991). In addition, some living microorganisms such as *Methanobacterium thermoautotrophicum* and *Methanobacterium thermoalcaliphilum* produce hydrocarbons, some of which are released to the atmosphere (Laurinavichyus *et al.*, 1988).

2.1.1.3 Environmental Effects of Hydrocarbon Pollution

Oil pollutants have contaminated marine, fresh-water, and soil ecosystems threatening human health and damaging the environment (Riser-Roberts, 1998; Gutnick and Rosenberg, 1977). The toxicity of crude and refined oil to marine ecology and even

more directly to man is a well-documented phenomenon (Boesch *et al.*, 1987). Suffice it to mention that crude oil contains mutagenic, carcinogenic, and growth-inhibiting chemicals, and even small quantities (5-100 μg L^{-1}) of certain petroleum fractions can destroy microalgae and juvenile forms of many marine organisms (Gutnick and Rosenberg, 1977). In short, oil pollution in general presents a serious problem to commercial fisheries, recreational resources, and public health (Boesch *et al.*, 1987).

2.1.1.4 Advantages of Bioremediation

The use of microbial biodegradative abilities in restoration of oil-polluted environments has been proven to have advantages over conventional treatments, offering a low-cost permanent solution and a much safer alternative in removal of oil pollutants (Atlas and Bartha, 1992). The cost effectiveness emerges through elimination of transportation expenses by treating oil pollutants on-site and by using biological systems which are very cheap compared to some chemicals used by traditional remediation techniques. The preference for using bioremediation over traditional methods also arises from the safety of its ultimate end products, CO_2 and biomass. Traditional methods, such as incineration, tend to create new waste often more harmful than the initial substrate.

2.1.2 Bioprocessing of Petroleum Hydrocarbons

Microorganisms possess oxidative enzymes capable of degrading various types of hydrocarbons for assuring their carbon and energy requirements and concomitantly transforming them into valuable products through series of reactions

(Baker and Herson, 1994). Many studies have been performed to determine the metabolic pathways for degradation of petroleum hydrocarbons. Members of each of the petroleum classes are metabolized at different rates, and some, for example, the asphaltic components of road tars, are either not metabolized or are metabolized so slowly as to make the rates negligible. The *n*-alkanes are considered to be the most readily degraded of all hydrocarbons; *n*-alkanes as long as 44 carbons can be metabolized by mixed populations of soil organisms (Britton, 1984). Branching generally makes an alkane more resistant to biodegradation (Pirnik *et al.*, 1984). Cycloalkanes are more refractory and some condensed cycloalkanes are extremely resistant, and therefore persist for very long periods in the environment (Perry, 1977). In olefins, the presence of one or more double bonds makes them more resistant to biodegradation (Watkinson and Morgan, 1990). Aromatic compounds are degraded, but less readily as the number of condensed aromatic rings increases. However, there have been some reports of low molecular weight aromatics that were attacked more rapidly than the alkanes (Fedorak and Westlake, 1981). The asphaltic fraction is most resistant to biodegradation and is the most persistent in different ecosystems. Relatively little is known of the metabolism of individual compounds in this fraction. Gibson (1984) has provided brief reviews of the degradation of individual hydrocarbons in petroleum.

2.1.2.1 Aliphatic Hydrocarbon Biodegradation

The biodegradation of *n*-alkanes and branched alkanes normally proceeds by a monoterminal attack; usually a primary alcohol is formed followed by an aldehyde and a monocarboxylic acid (ZoBell, 1950; Ratledge, 1978). Further degradation of the

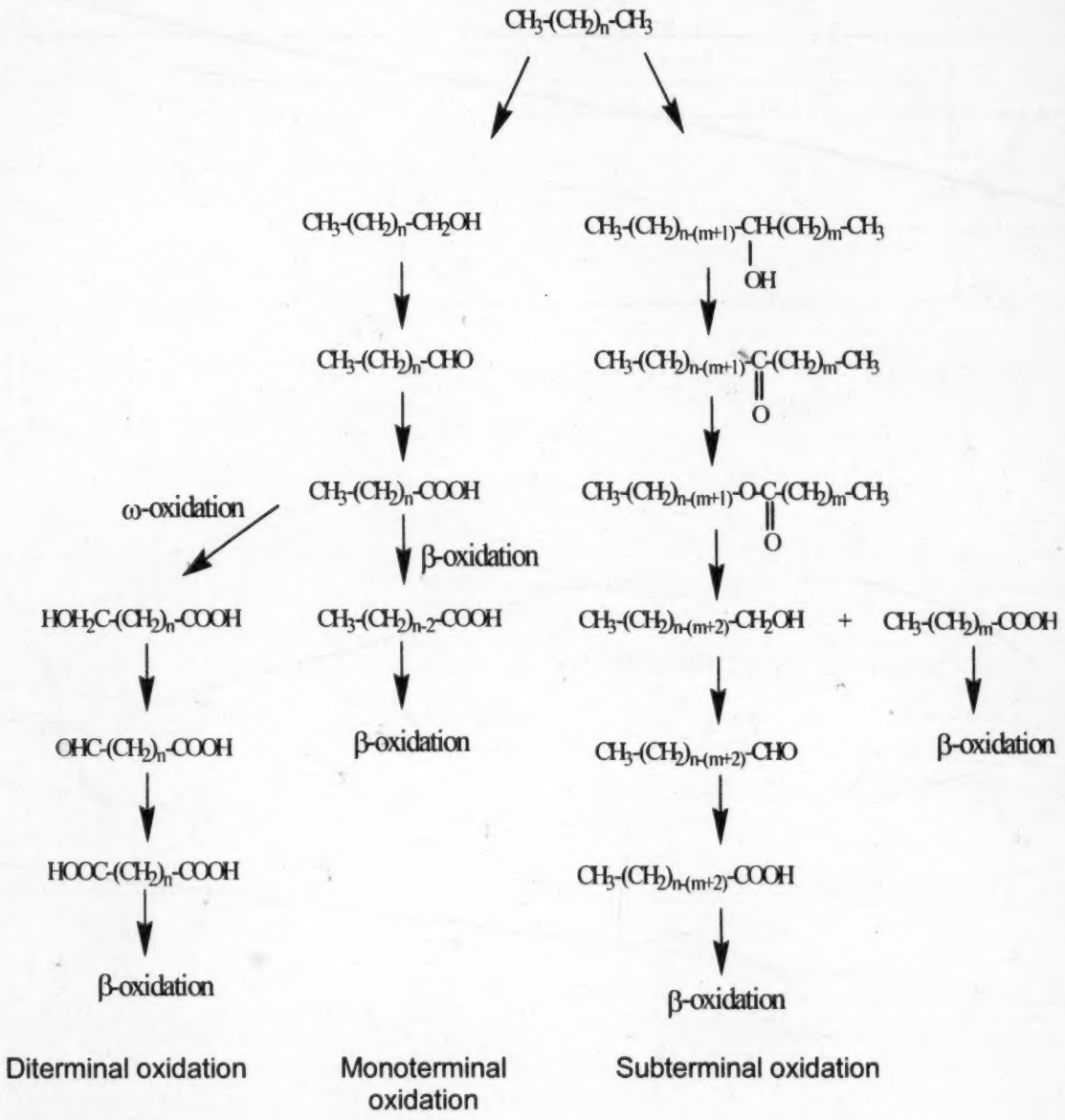
carboxylic acid proceeds by β-oxidation with the subsequent formation of two-carbon-unit-shorter fatty acids.

If β-oxidation is hindered by branching, the fatty acid is attacked at the other terminal carbon (ω-terminus) by the process called ω-oxidation (Jurtshuk and Cardini, 1971). The ω-terminus is progressively oxidized to an alcohol, aldehyde and carboxyl group. The resulting dicarboxylic acid can be further degraded by β-oxidation. The very intriguing oxidation, the diterminal oxidation, was also observed by Kester and Foster (1963) with a species of *Corynebacterium* and confirmed by Ali Khan *et al.* (1963) with a strain of *Pseudomonas*. Evidence suggests monoterminal oxidation, probably by hydroperoxidation, followed by ω -oxidation. Further breakdown occurs by β -oxidation at either end of the molecule. At that time, α, ω dicarboxylic acid production seemed to be an exciting discovery from a commercial point of view because it would make it possible to prepare a whole scheme of dibasic acids for polymerization purposes (Ali Khan *et al.*, 1963). Currently, α, ω dicarboxylic acids, considered versatile chemical intermediates used as raw materials for the preparation of perfumes, polymers, adhesives, and antibiotics, are obtained using *Candida tropicalis* strains (Craft *et al.*, 2003).

Subterminal oxidation sometimes occurs, with formation of a secondary alcohol and subsequently ketone, but this does not appear to be the primary metabolic pathway utilized by most *n*-alkane-utilizing microorganisms (Markovetz, 1971).

Highly branched alkanes, such as pristane, have been found to undergo ω oxidation, with formation of dicarboxylic acids as the major degradative pathway (Pirnik, 1977). Figure 2.1 presents a schematic of the possible *n*-alkane degradation pathways.

Figure 2.1: Degradative pathways of *n*-paraffins. The symbols *n* and *m* stand for a given number of CH_2 groups. Left, diterminal omega-oxidation; center, monoterminal beta-oxidation, and right, subterminal oxidation (Watkinson and Morgan, 1990)



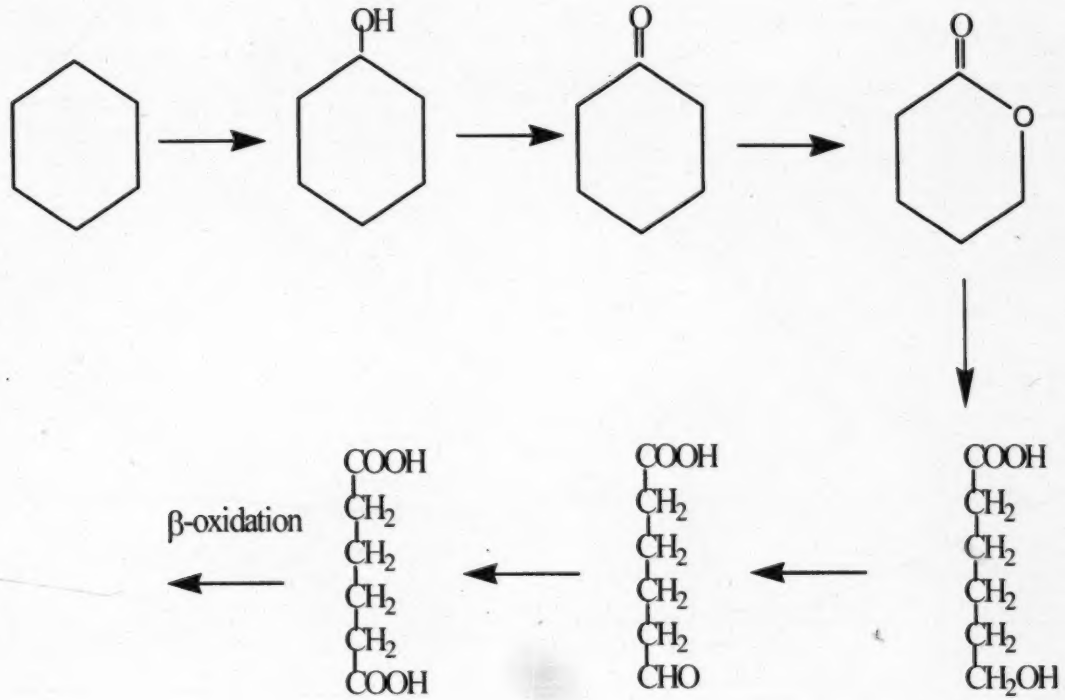
Alkenes may be attacked, either as the alkanes at a saturated terminal carbon, or may be oxidized directly at the double bond with formation of an epoxy compound. The epoxy intermediates are converted to diols, and then one of the hydroxyl groups is oxidized to a carboxyl, resulting in cleavage to a fatty acid and a primary alcohol (Watkinson and Morgan, 1990).

2.1.2.2 Cycloalkane Biodegradation

Cycloalkanes have been reported to be substrates for co-oxidation with formation of a ketone or alcohol (Perry, 1984). Once oxygenated, degradation can proceed with ring cleavage. Cycloalkanes metabolism appears to occur primarily through cometabolism followed by commensal utilization of the products by the other microbial strains (Perry, 1984). Figure 2.2 presents a schematic of cycloalkane biodegradation.

Alkyl-substituted cycloalkanes are generally attacked at the terminal (omega) alkyl carbon, which is oxidized to a carboxyl group. The resulting cycloalkylcarboxylic acids are metabolized by β-oxidation. An even number of carbons in the alkyl chain leads to cycloalkylacetic acid that is not readily utilized further. An odd number of carbons in the alkyl side chain leads to cycloalkylcarboxylate that can be metabolized further by dehydrogenation and partial aromatization.

Figure 2.2: Microbial oxidation of cyclohexane as an example for metabolism of alicyclic hydrocarbons (Perry, 1984)

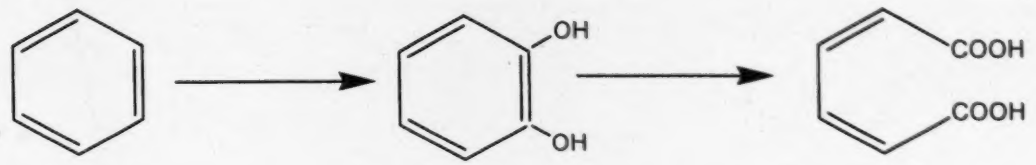


2.1.2.3 Aromatic and Condensed Polyaromatic Biodegradation

The degradation of aromatic hydrocarbons (e.g. benzene) normally involves the formation of a diol followed by cleavage and formation of a diacid such as *cis, cis* - muconic acid (Figure 2.3). One, two and three ring aromatics are readily degraded, and there are now several strains capable of degrading up to five-ring aromatic compounds (Cerniglia, 1992). *Mycobacteria* have been isolated that degrade 4-ring polynuclear aromatic hydrocarbons (Heitkamp and Cerniglia, 1989).

Two- and three-ring condensed aromatic hydrocarbons such as naphthalene, anthracene, and phenanthrene are degraded by successive opening of the aromatic rings, essentially by the mechanism described for benzene (Cerniglia, 1992, Bagy *et al.*, 1992). Alkylaromatic hydrocarbons with short alkyl moieties, such as toluene, can be also degraded by the mechanisms described for benzene. Alternatively, the initial attack may occur at the methyl group with a conversion, in several steps, to benzoic acid. Phenylalkanes with long alkyl chains are regularly metabolized starting at the terminal carbon of the alkyl moiety (omega oxidation).

Figure 2.3: Microbial metabolism of the aromatic ring (Cerniglia, 1984)



2.1.2.4 Asphaltic Components Biodegradation

The metabolic pathways for the degradation of asphaltic components of petroleum are probably least well understood. Asphaltenes are complex structures, which are difficult to analyze with current chemical methodology. The degradation of various sulfur-containing components of petroleum has been examined, but no uniform degradative pathway comparable to the pathways established for aliphatic and aromatic hydrocarbons has yet emerged for the asphaltic petroleum components. Practical experience shows that these compounds are highly resistant to biodegradation. Bertrand *et al.* (1983) found that the microbial degradation of asphaltenes and resins, which had previously been considered relatively recalcitrant to biodegradation, may be due to cooxidation. Cooxidation has been described by Raymond *et al.* (1970) as the phenomenon whereby the actively growing microbes oxidize a compound, but do not utilize either carbon or energy derived from the oxidation. Rontani *et al.* (1985) reported degradation of asphaltenic compounds in mixed bacterial cultures to be dependent upon the presence of long chain *n*-alkanes.

2.2 MECHANISMS OF HYDROCARBON TRANSPORT

A major question which arises in the biodegradation of hydrocarbons is how the microbes actually contact the substrates. Transport of hydrocarbons to the cells is a precondition for their metabolism. Unlike water-soluble growth substrates, which maintain constant contact with cells, water-immiscible alkanes must be transported to the cells in some way so as to achieve cell-substrate contact. Accordingly, a hydrocarbon-utilizing microorganism must possess a mechanism for the uptake of the hydrophobic, water-insoluble alkane across the cell envelope to the intracellular site of alkane oxidation (Singer and Finnerty, 1984).

At least two possible mechanisms for hydrocarbon transport have been invoked so far (Beal and Betts, 2000), and different microbial species may use one or more of them: direct contact with drops or surfaces of the insoluble phase and release of extracellular surfactants or emulsifiers that greatly increase the hydrocarbon-aqueous interfacial area. It has been reported that during growth of *Candida lipolytica* on *n*-hexadecane, both mechanisms played an equally important role in substrate uptake (Nakahara *et al.*, 1977). In the case of the hydrocarbon-degrading *Acinetobacter venetianus* RAG-1 both types of interactions are also operative; cells adhere avidly to test hydrocarbons (xylene, octane, hexadecane and crude oil) (Rosenberg *et al.*, 1980; Rosenberg *et al.*, 1982), and also produce a potent emulsifier referred to as emulsan (Zuckerberg *et al.*, 1979).

2.2.1 Direct Contact between Microbial Cells and Hydrocarbon Pollutants

Johnson (1964) suggested that growth on long chain liquid *n*-alkanes takes place through direct contact of liquid alkane and microbial cells. The organisms attach to the surface of hydrocarbon drops much larger than the cells, and substrate uptake presumably takes place through diffusion or active transport at the point of contact. Considerable evidence has been published since then supporting the hyphothesis that direct contact between the microorganisms and the hydrocarbon-pollutants is one of the main mechanisms by which bacteria interact with hydrophobic pollutants (Neufeld *et al.*, 1983; Goswami and Singh, 1991; Bouchez-Naitali *et al.*, 2001). Thus, phase microscopy of exponentially growing cells of *Acinetobacter* sp. has demonstrated a direct contact between the cells and the oil droplets (Baldi *et al.*, 1999; Rosenberg *et al.*, 1980). Kennedy *et al.* (1975) used phase contrast and scanning electron microscopy to observe the attachment of *Acinetobacter* sp. H01-N to the surface of alkane droplets; *Escherichia coli, Bacillus subtilis,* and *Enterobacter aerogenes* did not attach to alkane.

2.2.2 Bioemulsifier and Biosurfactant Production

Certain investigators have argued that microbial growth on hydrocarbons is mainly due to release of bioemulsifiers, which bring about emulsification of the hydrophobic substrate, rather than by direct contact between cells and hydrocarbon droplets (Wang, 1972; Pines *et al.*, 1986). Bioemulsifiers are high molecular weight products composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins or

complex mixtures of these biopolymers. They do not lower interfacial tension (Neufeld and Zajic, 1984; Ron and Rosenberg, 2002) but their presence at the interface makes them excellent candidates for emulsion stabilizers. By attaching to the newly created oil-water interface, they form a stable film around the oil droplet and prevent coalescence.

The best studied of these biopolymers is emulsan, the bioemulsifier produced by the oil-degrading bacterium *A. venetianus* RAG-1 (Zajic and Supplisson, 1972; Rosenberg and Ron, 1998). Emulsan is water-soluble, stabilizes a wide variety of hydrocarbon-in-water emulsions, and presumably binds tightly to the surface of hydrocarbon droplets. Whereas series of reports describe emulsan presence as favorable for hydrocarbon emulsification, others mention the hindrance of cell adhesion to hydrocarbons due to emulsan (Pines and Gutnick, 1986, Rosenberg *et al.*, 1983). *A. venetianus* RAG-1 produces emulsan whether it grows on soluble or insoluble substrates suggesting that emulsan production is not a requirement for growth on insoluble substrates to enable the contact between the microbial cells and the hydrocarbons (Neufeld and Zajic, 1984), and that microbes probably use another mechanisms for coming into contact with the hydrocarbon phase.

Another proposed pathway for making hydrocarbons available to the microbial cells was via biosurfactant production (Ron and Rosenberg, 2002). Biosurfactants include low molecular weight compounds produced extracellularly, namely glycolipids in which carbohydrates are attached to long-chain aliphatic acids or lipopeptides (Ron and Rosenberg, 2002). Examples of glycolipid biosurfactants produced by microorganisms include rhamnolipids, sophorolipids, and trehalose lipids (Pines and Gutnick, 1986; Desai and Banat, 1997; Zajic and Seffens, 1984). Being amphiphilic molecules, biosurfactants consist of a hydrophilic and a hydrophobic domain; the hydrophobic domain is a hydrocarbon whereas the hydrophilic group can be non-ionic, positively or negatively charged, or amphoteric (Finnerty, 1994). Because of

the presence of hydrophilic and hydrophobic groups within the same molecule, surfactants partition preferentially at the interface between the two fluid phases of different degrees of polarity (such as oil/water interface). The formation of an ordered molecular film at the interface lowers the interfacial energy and is responsible for the unique properties of surfactant molecules. One of the best-studied glycolipids is rhamnolipid, produced by several species of *Pseudomonas*, which consists of rhamnose and β-hydroxydecanoic acid (Hisatsuka *et al.*, 1971; Lang and Wullbrandt, 1999; Hommel, 1990), Figure 2.4. Another important glycolipid is the trehalose lipid of *Rhodococcus erythropolis* (Ristau and Wagner, 1983), Figure 2.5.

A surfactant is characterized by the critical micelle concentration (CMC), which in effect represents the solubility of a surfactant within an aqueous phase. At concentrations above the CMC, amphiphilic molecules associate readily to form supramolecular structures such as micelles, bilayers and vesicles, which act as "transport packets" for hydrocarbons, facilitating the hydrocarbon transfer to the cell surface. Since no chemical bonds are formed, these structures are fluid-like and are easily transformed from one state to another (Georgiou *et al.*, 1992). The interfacial tension between the aqueous and oleic phases changes very little above the critical micelle concentration because all additional surfactant molecules form micellar structures since the oil-water interface already has a monomolecular layer of amphiphiles. The formation of micelles can result in the solubilization of oil or water in the other phase, giving rise to a microemulsion.

Biosurfactants are usually produced by microorganisms in response to growth on hydrocarbons (Rapp *et al.*, 1979; Hommel, 1990) although there have been a few

Figure 2.4: Rhamnolipid of *Pseudomonas*; R_1 represents L-*α*-Rhamnopyranosyl and R_2 represents *β*-Hydroxy-decanoic acid (Hommel, 1990)

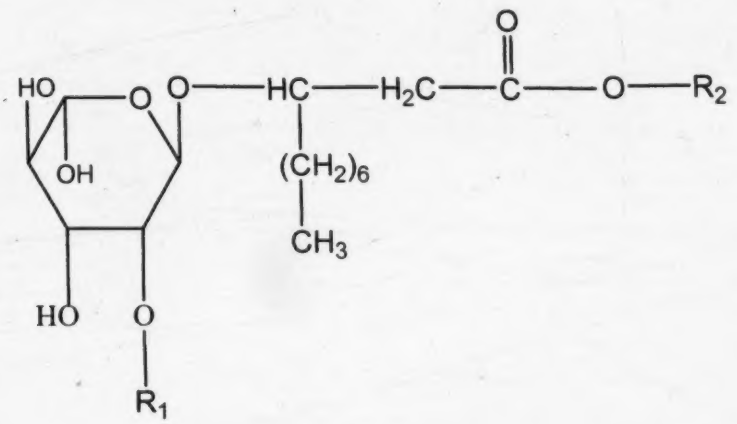
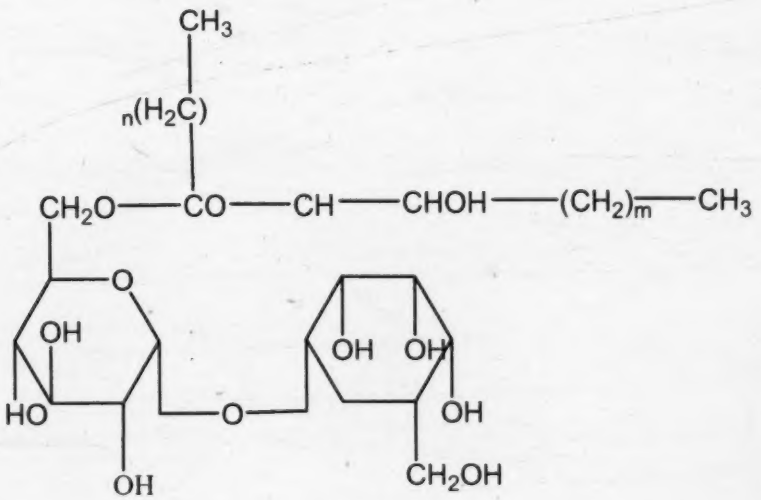


Figure 2.5: Trehalose of *Rhodococcus erythropolis* (Hommel, 1990)



examples of surface-active glycolipids produced on glucose (Cooper *et al.*, 1982; Mulligan *et al.*, 1984). Some bacteria such as *Pseudomonas aeruginosa* produce biosurfactants whether grown with water-soluble or water-insoluble substrates (Robert *et al.*, 1989). Another issue subject to debate in the literature is why the biosurfactant production is in some cases maximal on glucose, where there seems no need for the biosurfactant, and much lower on hydrocarbons, where it would seem to be beneficial (Prince, 1993).

Even though the above examples show that biosurfactants and bioemulsifiers are chemically two different kinds of products (Cooper, 1986; Hommel, 1990; Van Dyke *et al.*, 1991), which act on the insoluble substrate in different ways, the two terms have been sometimes mistakenly interchanged without taking into account the differences between them (Bento and Gaylarde, 1996; Schmid *et al.*, 1998); therefore the nomenclature in this area is sometimes confusing and lacks consistency.

In short, secretion of bioemulsifiers (Marin *et al.*, 1996; Bredholt *et al.*, 1997) or biosurfactants (Zajic and Seffens, 1984; Mulligan *et al.*, 1984) enhances hydrocarbon bioavailability to the microbial cell by dispersing the oil as fine droplets in an emulsion, thereby increasing the interfacial area between the oil and the water (Rosenberg, 1993), or increasing the apparent solubility of the oil in the aqueous phase (Cooper, 1986). Not all hydrocarbon degrading strains are dependent on production of bioemulsifiers or biosurfactants to be able to degrade hydrocarbons (Rapp *et al.*, 1979; Allen *et al.*, 1992).

2.3 GENERAL EMULSION CHARACTERISTICS

It has long been known that two immiscible, pure liquids cannot form an emulsion. For a dispersion of one liquid in another to be stable enough to be classified as an emulsion, a third component must be present to stabilize the system. As a result, emulsions can be defined as complex fluid dispersions formed and stabilized by adsorbed surfactants, polymers, proteins, finely divided solid particles, or their mixtures onto fluid-fluid interfaces (Mittal and Kumar, 2000). The adsorbed compounds protect the newly formed drops from re-coalescence.

Emulsions have been known from antiquity, the Greek physician Gale being apparently the first person to record the emulsifying power of beeswax (Becher, 2001). In spite of the early recognition of emulsions, it was only at the beginning of the last century when the distinction between the common forms of emulsions was made (water-in-oil or oil-in-water). It was soon realized that the type of emulsion which formed in a given mixture depended to a large extent on the choice of emulsifying agent.

The formation of an emulsion involves an increase of interfacial area and therefore an increase in free energy of the system. Thus an emulsion represents a state of higher free energy than that corresponding to the bulk components; therefore, transition to a state of lower free energy will tend to occur spontaneously unless there is a substantial energy barrier preventing droplets from coalescence.

Liquids tend to minimize their surface area; therefore, an emulsifying agent and work are required for emulsification to proceed. To break up an emulsion drop into smaller ones, it must be strongly deformed and any deformation increases pressure.

Consequently, the stress needed to deform the drop is higher for a smaller drop. Since the stress is generally transmitted by the surrounding liquid via agitation, higher stresses need more vigorous agitation, hence more energy (Gelot, 1984).

Two types of emulsion are now readily distinguished depending upon which kind of liquid forms the continuous phase: oil-in-water (O/W) for oil droplets dispersed in water and water-in-oil (W/O) for water droplets dispersed in oil. Whether an emulsion is of the W/O or O/W type depends on the tendency of the interfacial region to bend in one direction or the other (de Gennes and Taupin, 1982), which is determined by factors such as the difference in interfacial tension or interfacial pressure on the two sides of the interface, and the difference in the volumes and compressibilities of the hydrophilic and hydrophobic groups. If the hydrophilic side of the interfacial film has a greater tendency to expand than does the hydrophobic side, an O/W emulsion forms; in the converse case, if the hydrophobic side of the interfacial film has a greater tendency to expand than does the hydrophilic side, a W/O emulsion forms.

2.3.1 The Interface

In simple two-phase colloidal systems, a thin intermediate region or boundary, known as the interface, lies between the dispersed and dispersing phases. The interface determines the characteristics and behavior of the entire system (Schramm, 1992). The properties of the interface are dependent on the properties and interactions of the phases involved. Interfacial properties are very important because emulsified droplets have a large interfacial area, and even a modest interfacial energy per unit area can become a considerable total interfacial energy to be accommodated.

The requirement for a stable interface to form is that the free energy of formation of the interface should be positive. Negative or zero interfacial free energies will result in the complete dispersion of one phase into the other as in the case of two miscible liquids (Adamson and Gast, 1997).

2.3.2 Physical Nature of the Interfacial Film

The droplets of dispersed liquid in an emulsion are in constant motion and therefore there are frequent collisions between them. If, on collision, the interfacial film surrounding the two colliding droplets in a macroemulsion ruptures, the two droplets will coalesce to form a larger one, this phenomenon resulting in a decrease in the free energy of the system (Binks, 1998). The mechanical strength of the interfacial film is therefore one of the prime factors determining emulsion stability. As two emulsion drops approach each other, the thin liquid film between the drop surfaces must necessarily "drain out" before any coalescence can occur. With adsorbed emulsifiers at the oil-water interface, the drainage process may be significantly impeded due to the so-called *Marangoni effect*. (As the liquid film drains, the adsorbed molecules are dragged along the interface. The resulting non-uniform distribution of emulsifiers will be resisted by the "Gibbs elasticity," which tends to restore the emulsifier surface density to a uniform value (Mollet *et al.*, 2001)). The lifetime or stability of an emulsion film is determined primarily by its rate of thinning.

2.3.3 Mechanisms Involved in Emulsion Stability

Emulsion stability refers to the resistance of emulsions to the coalescence of their dispersed droplets. For a stable emulsion to form between two liquids, three basic conditions must be met: (1) the two liquids must be immiscible or mutually insoluble in each other; (2) sufficient agitation must be applied to disperse one liquid into the other; and (3) an emulsifying agent or combination of emulsifiers must be present (Mollet and Grubenmann, 2001).

The mechanism involved in emulsion stabilization and the basic origin of stability or instability of emulsions has been the subject of enormous debate in literature (Binks, 1998). Two mechanisms are believed to be responsible for emulsion stabilization, namely steric stabilization or physical barriers against coalescence and charge stabilization or electrical repulsion between droplets.

2.3.3.1 Steric Stabilization by Finely Divided Solids

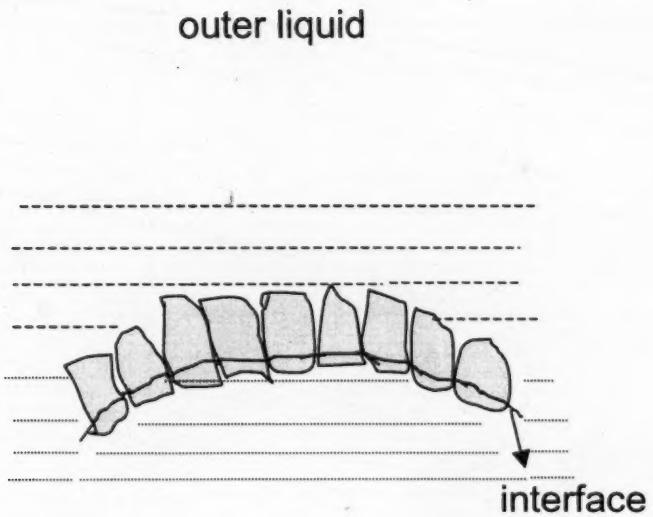
It has long been known that very effective stabilization against coalescence can be obtained by using finely divided solids as emulsifying agents. A number of investigators have prepared emulsions by using finely divided solids, which are insoluble in either phase, and do not affect the interfacial tension (Berkman and Egloff, 1941). It is readily understood that solids whose use results in stabilized emulsions must be, in some sense, amphipathic. Not, of course, in the same way as are surface-active agents, but rather in the sense that they are, to some extent, wetted by both the oil and aqueous phases (Becher, 2001). In the presence of suitable organic molecules, fine solids can be

adsorbed as a stabilizing film at the interface of oil/water emulsion droplets. Emulsion stabilization by solid particles depends partly on the ability of the particles to diffuse to the interfacial region and remain there in a state of mechanical equilibrium (Tambe and Sharma, 1992). At the interface, these fine particles usually form rigid structures that can sterically inhibit the coalescence of emulsion droplets. The experimental observations of Levine *et al.* (1989) that the emulsion droplets are extremely stable due to the close-packed monolayer of particles which act as a steric barrier to coalescence have been confirmed by other authors. Menon and Wasan (1988) also reported that the stability of O/W emulsions stabilized by clay particles depended on the formation of a rigid and protective structure at the oil-water interface. Such structures effectively sustain the particles at the oil-water interface.

Figure 2.6 shows the accumulation of finely divided solids at the oil/water phase boundary and formation of an interfacial "film" of high stability and firmness, which prevents the inner phase from coalescing thus increasing the stability of the emulsion.

There are two conditions which must be met before emulsion stabilization by particles can occur (1) the particle size must be much smaller than the droplet size and (2) the particle surfaces must be partially wettable by both oil and water (Levine *et al.*, 1989). The latter condition implies that the contact angle of the three-phase oil/water/solid system must be appreciably greater than 0° and less than 180°. The knowledge that emulsion stability is dependent on the wettability of finely divided solids dates back to the beginning of the last century when Pickering originally noted that colloids particles that were wetted more by water than oil could act as an emulsifying

Figure 2.6: Stabilization of an emulsion by adsorption of finely divided solids at the oil/water interface (Mollet and Grubenmann, 2001)



agent for oil-in-water emulsions (Pickering, 1907). Pickering (1907) found that basic sulfates of iron, copper, nickel, zinc, and aluminum act as efficient dispersing agents for the preparation of petroleum oil-in-water emulsions. Since then, several investigators have studied the stability of solid-stabilized emulsions. Briggs (1921) observed that silica stabilized oil-in-water emulsions while carbon black stabilized water-in-oil emulsions.

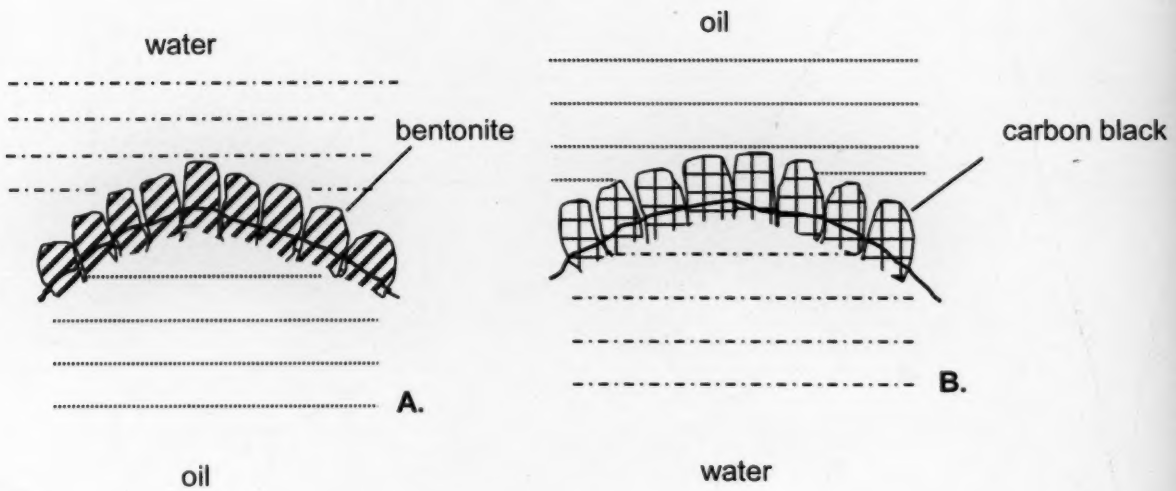
Figure 2.7 shows that the stability of the film bridged by the solid particles depends on the hydrophobicity of the particles expressed in terms of contact angle, θ, which the liquid surface makes with the finely divided solids. The solid particles (depending on their contact angle) can ultimately bridge the film by entry into both film surfaces. If the solid is preferentially wetted by one of the two phases, it can gather at the interface if this curves away from the wetting phase. In fact, finely divided solids are wetted principally by the outer phase. This results in the minimization of the interface between the two phases and thus in further stabilization of the droplets in the emulsion.

In short, the formation and stabilization of a solid-stabilized emulsion occurs in three stages: (1) approach of solid particles to the droplet surface, (2) entrainment of particles at the oil-water interface, and (3) interfacial stabilization by forming particulate networks. The first stage is a function of particle size, concentration and density. The second stage depends mainly on the three-phase contact angle. The formation of particulate networks at the interface is dependent on the magnitude of particle interactions (Menon and Wasan, 1988). The effect of each of these factors on the stability of an emulsion is presented in the following sections.

Figure 2.7: Stabilization of an emulsion by solid particles with different contact angles (Mollet and Grubenmann, 2001):

A. bentonite, a hydrophilic solid;

B. carbon black, a hydrophobic solid;



a). The Effect of Particle Concentration

Gelot *et al.* (1984) studied the effects of particle concentration on the stability of emulsions and reported an increase in emulsion stability, an increase in emulsion volume, and a decrease in the size of emulsion droplets as the concentration of particles available for emulsification is increased. A decrease in the size of emulsion droplets results in an increase in the total droplet surface area, and this enables additional particles to be accommodated at the interface (Tambe and Sharma, 1994). Obviously, a concentration of solids at the interface represents an interfacial film of considerable strength and stability, which serves to stabilize emulsions (Mollet and Grubenmann, 2001). This film formation is dependent on particle concentration, at low particle concentrations the emulsions produced are fairly unstable because there are insufficient particles to form an effective stabilizing film on the droplets created by agitation. On the other hand, when the particle concentration is increased, very stable emulsions are produced, because when more particles are available, a larger area of stable interface is formed.

b). The Effect of Particle Wettability

The wettability of solid particles is an important factor controlling the stability of the emulsion formed. Colloidal particles can effectively serve as emulsifying agents only if they are wetted partially by both the aqueous phase and the oleic phase (Tambe and Sharma, 1994). If there are enough solid particles to fill the interface, the tendency of the interface to contract will cause it to bend in the direction of the more poorly wetting

liquid, which makes it easy for the latter to become the dispersed phase. Schulman and Leja (1954) presented results in which they showed that solid particles that have contact angles less than 90° stabilized oil-in-water emulsions, while the particles that have contact angles greater than 90° stabilized water-in-oil emulsions. Yan *et al.* (2001) studied the role of fine silica particles in stability of oil/water emulsions by linking the type and stability of the emulsions formed to the three phase contact angle of the fine particles. The most stable emulsions occur when contact angle is close to 90°, so that the particles will collect at the interface. If the contact angle for a fine particle at the O/W interface is $\theta < 90°$, then most of the particle will reside in the aqueous phase; in this case an oil-in-water emulsion is indicated. Conversely, if $\theta > 90°$, then the particle will be mostly in the oil phase, and water-in-oil emulsion is predicted (Figure 2.8).

c). The Effect of Particle Size

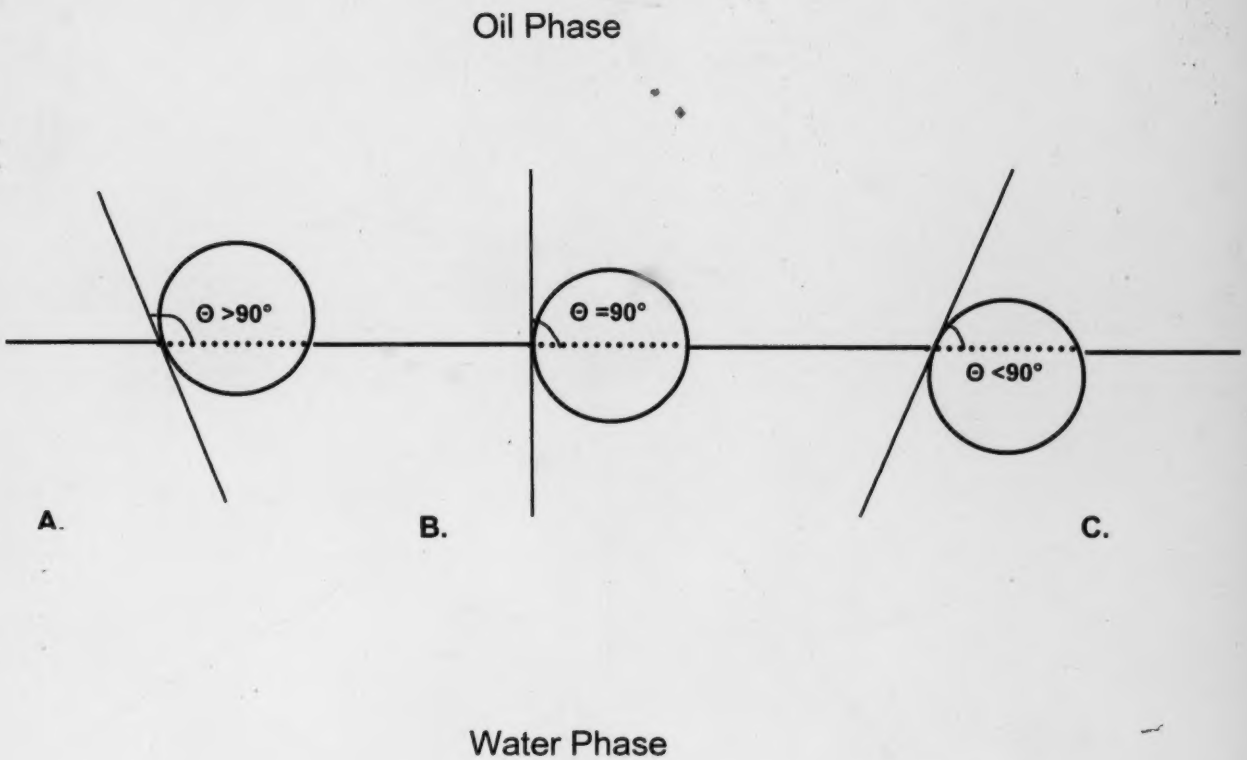
The effects of particle size on emulsion stability have been widely studied (Mollet and Grubenmann, 2001). The size of the particles used for emulsification may be one of the most important factors affecting the effectiveness of colloidal particles in stabilizing emulsions because it controls the ability of the particle to reside at the interface. It is necessary that the size of the particles be much smaller than the size of the emulsion droplets (Tambe and Sharma, 1994). This condition needs to be satisfied if the particles have to be properly located in a state of mechanical equilibrium around the droplets. Particles used for emulsion stabilization should, therefore, be in the order of a few microns. Tambe and Sharma (1994) showed that emulsion stability increased with a

Figure 2.8: The three ways in which finely divided solids may distribute themselves at the oil-water interface (Mollet and Grubenmann, 2001):

A. Solid wetted better by the oil than by the water;

B. Solid wetted equally by both phases;

C. Solid wetted better by the water than by the oil;



decrease in particle size until a critical particle size was reached. Below this critical size, Brownian effects were significant enough to affect the partitioning of particles at the oil-water interface. As a result, emulsion stability decreases as the sizes of the particles decrease below the critical size.

d). The Effect of Interparticle Interaction

Several investigators have reported that for effective stabilization to occur, solid particles need to be in a state of incipient flocculation (Binks, 1998; Mollet and Grubenmann, 2001). This observation clearly indicates that the interaction between particles is an important factor in the stabilization process. The strength of the interfacial film observed on the droplets of colloid-stabilized emulsions is a consequence of the degree of interparticle interactions (Menon and Wasan, 1988). The displacement of particles from the interface and hence coalescence is more difficult when particles attract one another.

2.3.3.2 Emulsions Stabilized by Electrical Repulsion

Most substances acquire a surface electric charge when brought into contact with a polar medium such as water (Adamson and Gast, 1997). The surface charge influences the distribution of nearby ions in the polar medium. Ions of opposite charge are attracted to the surface, but those with the same charge are repelled, forming an electric double layer. The electrical repulsive forces which arise when the double layers

surrounding charged droplets overlap is considered another mechanism responsible for emulsion stabilization; in this case the thickness of the double layer in relation to the size of the particle being an important parameter. The overall charge that a particle acquires in a medium is known as "zeta potential". If the emulsion's net zeta potential is greater than +30mV or less than -30mV the emulsion is relatively stable, and the particles possess enough charge to repulse each other. If the zeta potential is between these values, stability is poor (Mollet and Grubenmann, 2001).

2.3.4 Emulsion Instability

Emulsion instability may involve a number of processes, which take place simultaneously or consecutively, depending on conditions. The three main ways in which an emulsion may become unstable are creaming, flocculation, and coalescence (Binks, 1998).

There are reports in the literature of bacterial cell surfaces that can act as de-emulsifying agents of water-oil emulsions (Cairns *et al.*, 1983; Gray *et al.*, 1984). As an example, *Nocardia amarae* proved to be a capable de-emulsifying agent when compared to the commercial flocculating and de-emulsifying agents (Gray *et al.*, 1984). Whereas, for de-stabilizing a water-in-oil emulsion, younger cells of *N. amarae* were most effective, for de-stabilizing an oil-in-water emulsion, *N. amarea* cells from older cultures showed the most effectiveness (Stewart *et al.*, 1983). The common mechanisms responsible for emulsion de-stabilization are described in the following sections.

2.3.4.1 Emulsion Creaming

The buildup of a concentrated layer called cream in an emulsion system is well known and is a consequence of the density difference between the droplets and the continuous phase. Whereas in an oil-in-water emulsion the emulsion droplets move to form a concentrated layer at the top of the sample, in a water-in-oil emulsion the equivalent phenomenon moves downward and is called sedimentation. Creaming takes place without any change in the drop size distribution (Binks, 1998).

2.3.4.2 Emulsion Flocculation

The aggregation of emulsion drops to give three-dimensional clusters without rupture of the stabilizing layer at the interface is known as another way of destabilizing an emulsion. It may be weak (reversible) or strong (not easily reversible) depending on the strength of the interdrop forces (Binks, 1998). In aggregation the species retain their identity but lose their kinetic independence because the aggregate moves as a single unit.

2.3.4.3 Emulsion Coalescence

For coalescence to occur, the forces between the drop surfaces must be such that the film of continuous phase separating them can become sufficiently thin that film rupture becomes a likely possibility. Emulsion coalescence is an irreversible process. The rupture of the film is usually the result of thermal or mechanical fluctuation,

which results in stretching of the liquid surface with formation of surface waves that will grow in amplitude and result in droplet coalescence (Schramm, 1992).

2.3.5 Emulsion Gels

When the volume fraction of the dispersed phase of the emulsion exceeds the maximum packing volume fraction (where the droplets just touch each other), a gel emulsion is formed (Rajinder, 1999). This kind of emulsion contains tightly packed liquid droplets that form persistent microscopic liquid films between droplets and are characterized by yield stress, viscoelasticity and gel-like consistency (Babak and Stebe, 2002). Usually, the yield stress increases exponentially with the increase in the dispersed-phase concentration (Babak and Stebe, 2002). Gel emulsions are very important for different practical applications (Bampfield and Cooper, 1988), such as the food industry, cosmetics (Jager-Lezer *et al.*, 1998), protective films, topical drug delivery (Riess and Weers, 1996), production of low-density polymer foams for fusion-fuel capsules (Williams, 1991), and extraction of pollutants (Lye and Stuckey, 1998).

2.4 MICROBIAL EMULSIONS

Emulsions are often observed during bacterial growth on hydrocarbons and the benefit of their formation is the increase in interfacial area between oil and water, and therefore higher biodegradation rates (Cooney, 1984). Such emulsions are microbiological in origin and appear to be mediated via production of extracellular agents or by the cells themselves. After the bacteria adhere to the hydrocarbon surface, they

multiply on the surface. A short time later, the surface becomes saturated with bacteria, and growth becomes limited by the available surface. If the bacteria can split the oil droplets, emulsification occurs and new surfaces become available for growth (Rosenberg, 1993).

A similar phenomenon happens naturally when crude oil or petroleum products are spilled at sea. Indigenous microorganisms begin to degrade alkanes and small aromatic compounds almost as soon as the oil enters the water ecosystem (Leahy and Colwell, 1990). Both oil-in-water and water-in-oil emulsions may be formed as a result of wind and wave action. Whereas in an oil-in-water (O/W) emulsion oil droplets are dispersed and encapsulated within water column, in a water-in-oil (W/O) emulsion droplets of water are dispersed and encapsulated within the oil. The second mixture is generally referred to as mousse in the literature. In theory, the amount of energy required to increase the surface area can be calculated if the interfacial tension between the two liquids is known (Becher, 1955). In open ocean and coastal oil spills, sufficient energy to satisfy this requirement typically is provided by wind, waves, and currents. Nevertheless, stable water-in-oil emulsions also have been observed to form with certain oils even on very calm seas. In this situation the emulsifying agent may be responsible for emulsion formation by providing a thin interfacial film between the two liquids and maintaining the emulsion by minimizing the contact, coalescence, and aggregation of the internal dispersed phase (Payne and Phillips, 1985).

Mudd and Mudd (1924 a. and b.) described the behavior of microbes at a water/oil interface in a similar fashion to that developed by some authors for solid particles (Gelot *et al.*, 1984).

2.4.1 Emulsions Stabilized by Bacterial Extracellular Products

A number of organisms have been found to release extracellular bioemulsifiers into the culture broth, which brought about emulsification, a prerequisite for rapid biodegradation, giving large surface areas available for microbial activity (Wang, 1972; Pines *et al.*, 1986). Increasing the surface area is expected to increase the frequency of collision between cells and oil, as well as to provide increased surface for growth. However, Goswami and Singh (1991) noted that extracellular bioemulsifier may not be essential for substrate transport if cells firmly adhere to the surface of hydrocarbon drops as soon as these are generated through agitation-stirring.

2.4.2 Emulsions Stabilized by Bacterial Cells

Emulsification by intact bacterial cells instead of bio-products in the media is not a well-documented phenomenon (Allen *et al.*, 1992). While reports of microbes synthesizing extracellular surface active compounds during growth on hydrocarbons are numerous in the literature, there are only a few reports which consider the emulsifying capabilities of the whole cells (Cooper *et al.*, 1982; Neufeld and Zajic, 1984; Velankar, 1975; Allen *et al.*, 1992). Neufeld and Zajic (1984) reported that *Acinetobacter venetianus* sp. 2CA2 demonstrated the ability to emulsify and stabilize a kerosene-water emulsion. This ability was not related to the lowering of the liquid surface and interfacial tension, since both surface and nonsurface active cells demonstrated the same emulsifying properties. They also showed that both citrate and hexadecane-grown cells were equally effective as emulsifiers.

2.5 MICROBIAL CELL SURFACE PROPERTIES

Certain microorganisms can bring about emulsification of hydrocarbons even in the absence of cell growth (Rosenberg *et al.*, 1980; Cooper *et al.*, 1982; Rosenberg and Rosenberg, 1985), suggesting that emulsification is rather a cell-surface associated phenomenon, realized by general hydrophobic interactions more readily than specific recognition of the substrate. As an example, Rosenberg *et al.* (1980) demonstrated a high affinity of nutrient broth-grown *Acinetobacter venetianus* RAG-1 and BD 413 for hexadecane, octane and xylene; the last two hydrocarbons being nonmetabolizable by this bacterium, indicating that adherence to hydrocarbon is probably due to a nonspecific hydrophobic interaction. Moreover, that adherence to hydrocarbons is not restricted to hydrocarbon-degrading microorganisms was again emphasized by Rosenberg and Rosenberg in 1985, when they showed that various bacteria which do not metabolize hydrocarbons, including strains of *Staphylococcus* and *Streptococcus,* adhere avidly to hydrocarbons.

In explaining microbial adhesion to insoluble substrates and stabilization of oil/water emulsions, the microbial cell surface properties plays a very important role (Van Loosdrecht, 1987). For contact and adhesion to occur, it is necessary that there be some form of affinity between the cell surface and the hydrocarbon.

Hydrophobic surface properties of microbial cells have been extensively investigated starting with the pioneering observations by Mudd and Mudd (1924 a. and b.) of bacterial partitioning at oil-water interface. Bacterial surface hydrophobicity is involved in interfacial interactions of microbial cells with other microbial cells (flocculation) and with liquids and solids (Rosenberg and Kjelleberg, 1986). The microbial cell surface hydrophobicity is often evaluated by bacterial adhesion to

hydrocarbon test, BATH, (Rosenberg *et al.*, 1980), water contact angle measurements, CAM, (Busscher *et al.*, 1984; Neufeld *et al.*, 1980), hydrophobic interaction chromatography, HIC, (Ahimou *et al.* 2001), partitioning of cells into one or another liquid phase, TPP, (Albertsson, 1958), adhesion to polystyrene and other hydrophobic surfaces (Rosenberg, 1981), and salt agglutination test, SAT, (Lindahl *et al.*, 1981).

2.5.1 Bacterial Surface Structures

The surfaces of microbial cells are vital to the organisms' survival, since it is via their surfaces that the bacteria interact with the environment (Pembrey *et al.*, 1999). The cell walls of many bacterial species are surrounded by a polymeric substance referred to as a capsule (if discrete) or a slime layer (if amorphous) (Costerton *et al.*, 1987). Capsules are sticky, gelatinous structures composed of polymeric material and difficult to view.

It has become increasingly clear that the presence of capsules in almost all instances results in decreased adhesion and hydrophobicity. The presence of capsule has been reported to hinder adhesion to hydrocarbons by *Pseudomonas multicida* (Thies and Champlin, 1989), *Streptococcus pyogenes* (Ofek *et al.*, 1983), and *Acinetobacter venetianus* (Rosenberg *et al.*, 1983).

Attempts have been made to relate the absence or presence of a capsule with the hydrophobicity of staphylococci. Although the majority of encapsulated strains were hydrophobic (Hogt *et al.* 1985), there are reports of some nonencapsulated strains which are also hydrophobic (van der Mei *et al.*, 1989). Therefore, it cannot be concluded that there is a direct correlation between the presence of the capsule and

hydrophobicity. Even though it is believed that capsules can often be removed from organisms by simple centrifugation procedures, it has been reported that after several washings and centrifugations, *Azospirillum brasilense* cells, for example, still possess a capsule at all phases of growth (Yagoda-Shagam *et al.* 1988).

In addition to capsules, many bacterial surfaces possess one or more flagella, special structures that enable them to reach different regions of their microenvironment. Bacterial flagella are long, thin appendages, helically in shape, free at one end and attached to the cell at the other end (Brock and Madigan, 1991).

Other important cell surface structures are fimbriae and pili, which are structurally similar to flagella but are not involved in motility. Fimbriae are considerably shorter than flagella, more numerous, and they are believed to enable microorganisms to stick to inert surfaces, or to form pellicles on the surfaces of liquids (Brock and Madigan, 1991). Rosenberg *et al.* (1982) reported that adherence of *Acinetobacter calcoaceticus* RAG 1 to hexadecane may be due to the presence of numerous fimbriae on its surface. On the other hand, the strain RV 1 adhered relatively poorly to liquid hydrocarbons, even though it possessed an abundance of fimbriae on its surface (Rosenberg *et al.*, 1982). Pili are similar structurally to fimbriae but are generally longer and they are present in a much smaller number on bacterial surfaces. Pili are involved in attachment to human tissues and in the mating process in bacteria (Brock and Madigan, 1991).

2.5.2 Measurement of Surface Properties

a). Bacterial Adhesion to Hydrocarbon Test (BATH)

It has been observed for a long time that certain bacteria adhere to liquid hydrocarbons during petroleum fermentations (Neufeld *et al.*, 1980). From the need to quantify this observation, Rosenberg *et al.* (1980) developed an assay, which shows that various bacterial strains thought to possess hydrophobic surface characteristic adhere to liquid hydrocarbons, whereas non-hydrophobic strains do not. This method, based on the degree of adherence of cells to various liquid hydrocarbons, consists of combining certain volumes of aqueous cell suspensions with *n*-hexadecane followed by a brief period of mixing by vortex. After mixing, the two phases are allowed to separate and the turbidity of the lower, aqueous phase is determined. If the cells have a high cell surface hydrophobicity they will adhere to the hydrocarbon droplets and rise to the surface. Thereby causing a decrease in the turbidity of the aqueous phase (Rosenberg *et al.*, 1998). On the other hand, if the cells are nonadherent, the hydrocarbon droplets quickly coalesce, and the turbidity in the lower aqueous phase remains unchanged. There is presumably one drawback attributed to this technique, namely, the possibility that cells are damaged by vortexing in the presence of the liquid hydrocarbons (Rosenberg and Doyle, 1990). However, Vanhaeke and Pijck (1988) have shown that *n*-hexadecane does not damage the integrity of microbial cells during the assay. Pembrey *et al.* (1999) noticed that the loss or extraction of cell surface components during the mixing cell suspensions with *n*-hexadecane is rare.

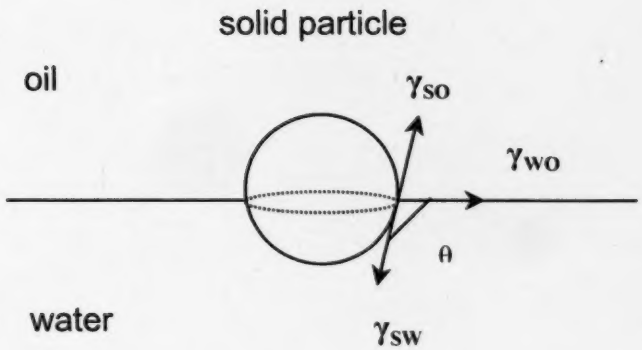
b). Contact Angle Measurement

Because of the impossibility of measuring surface tension of solid surfaces, considerable attention has been paid to alternate methods that quantitate hydrophobicity on the basis of measurement of contact angles (Gould, 1964). Contact angles can be measured readily on most solids and are often used simply as empirical parameters to quantify wettability.

When a droplet of oil in water comes into contact with a solid surface, the oil may form a bead on the surface, or it may spread and form a film. A liquid having a strong affinity for the solid will seek to maximize its contact (interfacial area) and form a film. A liquid with much weaker affinity may form into a bead. The affinity is termed wettability. The contact angle, θ, in an oil-water-solid system is defined as the angle, measured through the aqueous phase that is formed at the junction of the three phases (Figure 2.9). Whereas interfacial tension is defined for the boundary between two phases, the contact angle is defined for a three-phase junction. The solid is completely water-wetted if $\theta = 0°$ and only partially wetted otherwise. Complete non-wetting by water would mean that $\theta = 180°$. Also, values of $\theta < 90°$ are often considered to represent "water-wetting", and values of $\theta > 90°$ are considered to represent "non-water-wetting".

More than 180 years ago, Thomas Young proposed that the contact angle between a liquid and a solid arises from the requirement that the interfacial tensions acting at the three-phase line be balanced at equilibrium (Duncan-Hewitt, 1990). If the interfacial forces acting along the perimeter of the droplet are represented by the interfacial tensions, then an equilibrium force balance can be written as (2.1):

Figure 2.9: A spherical particle at an oil-water interface



$$\gamma_{W/O} \cos \theta = \gamma_{S/O} - \gamma_{W/S}$$ Young equation (2.1)

where the subscripts refer to water (W), oil (O), and soild (S). This equation is based on the hypothesis that the competition between the cohesive forces of a liquid and the adhesive forces between the liquid and a solid surface result in a contact angle which at equilibrium is constant and specific to the particular system. It should be noted that while $\gamma_{W/O}$ and θ may be determined experimentally with ease, the Young equation contains two unknowns, $\gamma_{S/O}$ and $\gamma_{W/S}$, which for years have eluded researchers (Mollet and Grubenmann, 2001). To complicate the situation, the equilibrium contact angle to which Young refers is difficult to determine, since contact angles show hysteresis between an advancing maximum and a receding minimum. The main causes of hysteresis are heterogeneity of the surface and roughness. Since some interactions are to be expected between an aqueous drop and a solid surface, an advancing contact angle should provide more useful information than a receding contact angle.

Contact angle is a classic method for measuring solid surface hydrophobicity, therefore, it has been extended to microbial cell surfaces, being considered the most suitable method in this regard so far (Krekeler *et al.* 1989). The measurement of contact angle of microbial cells presents difficulties which are not commonly encountered in most simple liquid and solid systems. The most extensive work in this area has been done by van Oss and Gillman (1972), who conducted contact angle measurements on phagocytic and other cells, as well as on various bacteria. Contact angles were determined using physiological saline on dried bacterial cells. The technique of measuring contact angles on dried cells has the advantage of simplicity, however,

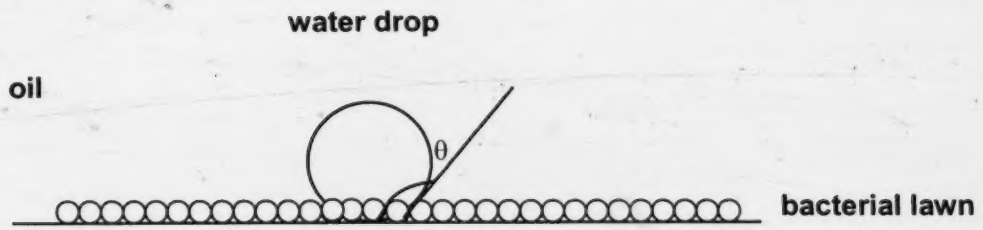
changes which occur during the drying of the cell surface have a very dramatic effect on the contact angle (Neufeld *et al.*, 1980). An additional problem which is encountered in hydrocarbon fermentation is that the cells are frequently adhering to, or immersed in the hydrocarbon phase, thus drying of the cells is difficult and any attempt to remove the adhering hydrocarbon from the cell surface would cause extensive damage to the cell (Neufeld *et al.*, 1980).

While most studies on the nature of bacterial surfaces have concentrated on the contact angle of liquids on bacterial layers in air, only a few have examined the wettability in a three-phase system involving the cell lawn and two immiscible liquids (El-Shimi and Goddard, 1974; Neufeld *et al.*, 1980). Neufeld *et al.* (1980) described a technique in which microbial cells were immersed in a hydrocarbon bath, and then the contact angle of an aqueous drop placed on the cell surface was determined (Figure 2.10). The advantage of this method is that cells do not have to be dried and, therefore, no denaturation of macromolecules on the cell surface occurs (Krekeler *et al.*, 1989). By measuring the hydrophobicity of living cells in an aqueous environment the *in vivo* situation can be approximated.

c). Measurement of Microbial Surface and Interfacial Tensions

The most fundamental thermodynamic property of any interface is the interfacial tension, yet its origin has been the cause of much confusion and controversy (Binks, 1998). Numerous techniques of interfacial tension measurement have been developed in the past, most common being the Wilhelmy plate and Du Nouy ring, which measure the force needed to traverse an object through an interface. However, these methods are effective only in measuring surface and interfacial tension between liquid-

Figure 2.10: Contact angle of sessile drop on bacterial lawn



gas and liquid-liquid systems, and they are not applicable to solids (van Loosdercht *et al.*, 1987). Whereas it is well known that solid surface tension cannot be measured, there are reports in the literature of interfacial tension measurements involving a three-phase system consisting of water, hydrocarbon and bacterial cells (Goswami and Singh, 1991; Whyte *et al.*, 1999).

Recently, a micropipette technique has been developed to measure the interfacial tension of micrometer-sized oil droplets covered by silica beads or clay particles (Yeung *et al.*, 2000; Chiang and Elliott, 2002). This technique, adapted from the field of biophysics, involves capturing individual emulsion drops (oil drops) at the tips of glass suction pipettes and measuring the pressure needed to break the emulsion droplet (Moran *et al.*, 1999). This method is based on the equation attributed to Young (1804) and Laplace (1805), that relates the pressure across a curved interface, Δp, to the interfacial tension, γ, and radius of curvature, R_c:

$$\Delta p = p_A - p_B = 2\gamma / R_c \qquad \text{Young-Laplace equation} \quad (2.2)$$

In an interface between phase A in a droplet and phase B surrounding the droplet, the phases will have pressures p_A and p_B. Interfacial tension causes a pressure difference to exist across a curved surface, the pressure being greater on the concave side (inside of a droplet). The interface is characterized by a single radius of curvature that is equivalent to the pipette radius only if the interface is a hemispherical cap in the pipette with zero contact angle at the pipette wall (Lee *et al.*, 2001). For a nonzero contact angle, the radius of curvature is greater than the pipette radius. Thus, the difference in pressure is inversely proportional to the radius of curvature of the interface. Then, for a

given pressure, if the radius of curvature can be measured, the interfacial tension can be determined from the Young-Laplace equation. For a straight pipette with one radius, there can only be one equilibrium point. However, and this is a key feature of this technique, in a tapered pipette, a continuum of equilibrium points can be obtained simply by changing the applied pressure and moving the interface to a new position in the taper (Lee *et al.*, 2001).

Neufeld and Zajic (1984) measured the surface tension of the whole culture broth of *Acinetobacter calcoaceticus* 2CA2 using a Du Nouy surface tensiometer and reported a decrease in surface tension with time from 50 mN/m, initially, to 27 mN/m after 25 h of growth. Whyte *et al.* (1999) have also reported the measurement of surface tension of a *Rhodococcus* sp. Q15 culture broth using a surface tensiomat. The decrease of the surface tension with time was recorded in this case, as well. In both these studies, when the cells were removed from the culture broth and the surface tension of the cell-free spent medium was measured, no decrease in the surface tension value was observed. As a result, the overall conclusion drawn from the above experiments was that bacterial cells exhibited surface activity, expressed through the reduction of surface tension of the whole culture broth. This conclusion is misleading since it has been known for a long time that solid surface tension can not be measured (van Loosdrecht *et al.*, 1987).

2.5.3 Comparison of Measurement Methods

A controversial issue is the degree of correlation between the various hydrophobicity techniques (Rosenberg and Doyle, 1990). The methods employed in measuring cell surface hydrophobicity have been subject to comparison (Mozes and

Rouxhet, 1987; van der Mei *et al.*, 1987); the outcomes of various tests correlate only for particular populations of microbial strains and they are still subject to questioning (van der Mei *et al.*, 1995; Ahimou *et al.*, 2001). Van der Mei *et al.* (1987) compared a range of *Streptococcus* isolates by using bacterial adhesion to hydrocarbon test, contact angle measurement, salt agglutination test and hydrophobic interaction chromatography. They found weak correlations among the various techniques.

From the above literature review, it is obvious that the examination of microbial behavior at the oil/water interface is a debatable and poorly understood subject. Therefore, the knowledge of solid particle (such as silica) behavior at the interface between oil and water enabled us to elaborate a few hypotheses regarding bacterial behavior when coming into contact with a two immiscible liquid system such as hydrocarbon and water. The hypotheses underlying this project were that bacterial cells stabilize oil-water emulsions by the same mechanism fine solids use in stabilizing oil-water emulsions (namely steric hindrance of coalescence) and microbial adhesion to the oil-water interface is dependent on cell surface hydrophobicity. This research project was approached by first determining cell surface hydrophobicity through different methods and then preparing emulsions to test the ability of different bacterial cells to form and stabilize emulsions. Finally, the correlation between cell surface hydrophobicity and bacterial ability to form and stabilize emulsions was determined and compared to results available for fine particles.

III MATERIALS AND METHODS

3.1 MICROBIOLOGICAL METHODS

3.1.1 Chemicals

n-Hexadecane (99% pure) was purchased from Sigma Chemical Co. (St. Louis, MO.), toluene (HPLC grade) and dichloromethane (HPLC grade) were purchased from Fisher Scientific. All other chemicals were reagent grade, unless otherwise stated, and purchased from Fisher Scientific.

3.1.2 Bacterial Strains

The alkane-degrading bacteria employed in this study were *Acinetobacter venetianus* RAG 1, *Rhodococcus erythropolis* 20S-E1-c, *Rhizomonas suberifaciens* EB2-1a, and *Pseudomonas fluorescens* LP6a. *A. venetianus* RAG-1 (ATCC 31012) was originally isolated for growth on crude oil by Reisfeld *et al.* (1972) from a marine beach. This bacterium was initially identified as a member of the genus *Arthrobacter,* renamed *Acinetobacter calcoaceticus,* and subsequently reclassified as a strain of *A. venetianus* (Bach *et al.*, 2003; Vaneechoutte, 1999). It grows on a variety of carbon sources including crude oil, pure aliphatic hydrocarbons, alcohols, organic acids, triglycerides, and other alkyl esters. *R. erythropolis* 20S-E1-c was isolated from a marine sediment in

Washington State (Foght, 1999), *R. suberifaciens* EB2-1a was isolated from a refinery wastewater in Germany (Foght *et al.*, 1998) and *P. fluorescens* LP6a was isolated from a hydrocarbon-contaminated soil in Alberta, Canada (Foght *et al.*, 1998).

3.1.3 Growth Conditions

The microbial growth medium used throughout the experiments was Trypticase Soy Broth from Difco Laboratories (TSB, Detroit, USA) which contained 17 g/L pancreatic digest of casein, 3 g/L papaic digest of soybean meal, 5 g/L sodium chloride, 2.5 g/L dipotassium phosphate, and dextrose. All media used in this study were prepared in distilled water, purified on a Milli-Q_{plus} apparatus (Millipore) to a resistivity higher than 18.2 M Ω cm^{-1}.

To prepare the microbial cultures for use, a vial of each microbial strain suspended in glycerol was removed from the freezer. The microorganisms were streaked on plates of Trypticase Soy Agar from Difco Laboratories (TSA, Detroit, USA), incubated at 28°C for 3 days and stored at 4°C. Fresh plates were prepared from glycerol stock as required.

The microorganisms were transferred from their respective agar plates, using a loop, into 250-ml Erlenmeyer flasks, plugged with cotton wool, and each containing 100 ml of autoclaved Trypticase Soy Broth (TSB, Detroit, USA). The flasks were then placed on an incubator-shaker (Innova 4000, New Brunswick Scientific) and incubated at 28°C with shaking at 200 rpm. The duration of incubation of all cultures was such that harvesting took place at the beginning of the stationary phase (Table 4.1). Cells were harvested by centrifugation at 11, 500 × *g* for 10 min and washed twice with phosphate buffer (pH=7). The cell suspensions used throughout this study were obtained by

resuspending bacteria in 100 mM phosphate buffer (pH 7). Cell densities are given for the specific subsequent experiments.

3.1.4 Biomass Determination

3.1.4.1 Optical Density

The optical density of different bacterial suspensions in phosphate buffer (pH 7) was measured at 600 nm using a spectrophotometer (SPECTRA max PLUS^{384}, Molecular Devices, Sunnyvale, CA). Before performing the experiments, the spectrophotometer was calibrated using phosphate buffer as a blank reference.

3.1.4.2 Cell Dry Weight

Samples of the whole cell broth were centrifuged at 11,500 × *g* and 4°C for 10 min. The supernatant or cell-free broth was decanted and the pellet washed twice with distilled water and resuspended in distilled water. Then sets of three 10 mL samples of bacterial suspensions in distilled water, with different optical densities, were spread on dry aluminum plates of known weight. The blank was a 10 mL distilled water sample dried at the same time interval of 24 hours in a 100°C oven. Later the dry samples were weighed and the weight of the blank was subtracted from the total weight. The resulting weight represented the dry weight corresponding to a certain optical density.

3.1.5 Bacterial Adhesion to Hydrocarbon Test (BATH):

A simple, rapid quantitative assay for the hydrophobic interaction of cells with liquid hydrocarbons was developed by Rosenberg *et al.* (1980) due to the large interest in the mechanism, which enables direct contact between hydrocarbon-degrading cells and their water-insoluble liquid alkane substrate. The original method was based on the degree of adherence of early logarithmic growth phase cells, suspended in a mixture of phosphate buffer, $MgSO_4$, and urea (PUM buffer), to various liquid hydrocarbons following a brief period of mixing. In our case, we used a modified bacterial adhesion to hydrocarbon assay to test the cell surface hydrophobicity of late stationary phase *A. venetianus* RAG 1, *R. erythropolis* 20S-E1-c, *R. suberifaciens* EB2-1a and *P. fluorescens* LP6a (suspended in phosphate buffer) with *n*-hexadecane. Stationary phase cells, grown on TSB, were harvested, washed twice with phosphate buffer at 4°C and resuspended in phosphate buffer. Cell pellets were suspended in phosphate buffer to give an optical density at 600 nm of about 0.6. Aliquots of cell suspension (1.2 mL) were transferred to acid-washed test tubes (10 mm i.d.) containing 1 mL of *n*-hexadecane. After incubation at 28°C for 10 min, the test tube contents were mixed by vortex for 120 s and allowed to separate for 15 min. The aqueous phase was carefully removed with a Pasteur pipette, transferred to a 1.5 mL cuvette and its OD_{600} was measured spectrophotometrically using a SPECTRA max PLUS^{384} (Molecular Devices, Sunnyvale, CA). The difference between the optical density of the aqueous phase before and after mixing with *n*-hexadecane was used to express hydrophobicity as a percentage: $100 \times \{1- (OD_{600}$ after mixing/ OD_{600} before mixing)$\}$. For a given sample, three independent determinations were made, with the error estimates giving a 95% confidence interval.

3.1.6 Capsule Staining

The capsule of bacteria does not have the same affinity for dyes as do other cell components, and this necessitates the use of special staining procedures. Capsules are fragile and easily disrupted by water and heat, therefore, staining bacterial capsules is not an easy process. The use of heat fixation may destroy or distort the capsule therefore air drying should be used instead.

For making the capsule visible we used Gin's Method (Pierce and Leboffe, 1999), which uses India Ink (Reeves & Poole Group Inc., ON.) to stain the background and Methylene Blue (Sigma Chemical Co., St. Louis, MO.) to stain the cell. The capsule appears as a white halo between the blue cell and the dark background. First, a loopful of India Ink was mixed with a loopful of sterile saline at one end of a clean microscope slide. Then, a very small amount of bacterial suspension was transferred and mixed, aseptically, into the diluted India Ink. Holding a second slide at a 45° angle, the end of the slide was touched to the suspension of bacteria and allowed the drop to spread along the angled slide. Without raising the slide the suspension drop was pulled across the slide forming a feathered film with gradations of darkness. In the next step, the angled slide was discarded and the sample slide was allowed to air dry thoroughly. Finally, the smear was flooded with Methylene Blue (0.05 wt. % in water) for 3 minutes, rinsed with distilled water and allowed to air dry for another 3 minutes. After the entire staining procedure was completed, the capsules were observed under a light microscope.

3.2 PHYSICAL MEASUREMENTS

3.2.1 Surface and Interfacial Tension Measurements of Cell-Free Spent Medium

To determine if any reduction in the surface properties occurred during bacterial growth in TSB medium, the cells were removed from the culture broth by filter sterilization and the surface activity of the cell-free spent medium was determined using a tensiometer (Single Fibre Tensiometer K14, Krüss, USA).

Surface tension measurements were conducted by lowering a platinum ring into the sample liquid. Then, a sensitive strain gauge from which the ring was suspended measured the force required to pull the ring through the sample surface. This force gave a direct measurement of the surface tension.

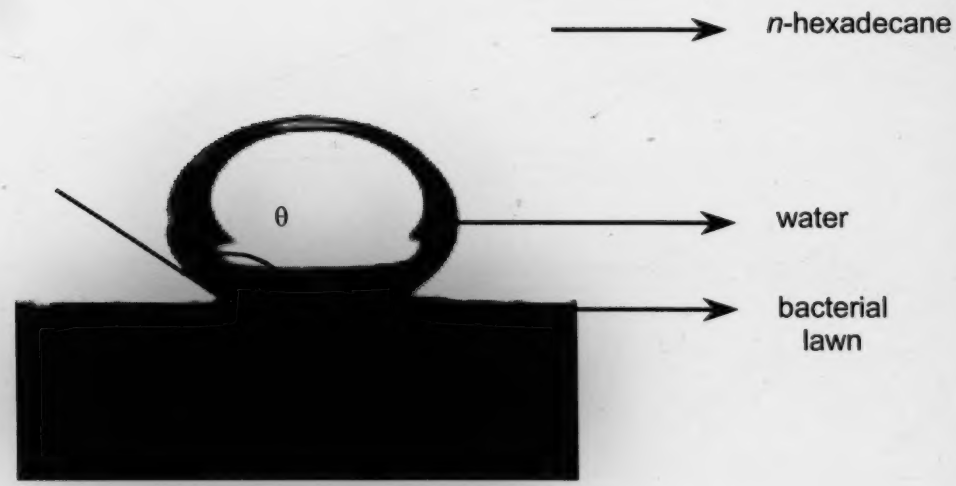
Interfacial tension between the sample liquid and *n*-hexadecane was determined by lowering the platinum ring into the aqueous sample and then gently layering an equal volume of hydrocarbon on the surface. The force required to pull the ring through the aqueous-oil interface gave the interfacial tension. All measurements were done with fresh interfaces at room temperature; each reported value is the mean of 3 independent measurements and resulted in a standard deviation about the mean of approximately 2°.

3.2.2 Contact Angle Measurements

The test was performed using a modified protocol from Neufeld *et al.* (1980). Bacteria were first harvested by centrifugation (11,500 × *g*, 4°C, 10 min), washed twice with phosphate buffer (pH 7) and resuspended in phosphate buffer (pH 7). A lawn of bacterial cells was then prepared by filtering cell suspensions in phosphate buffer through a 0.22 µm pore size PVDF membrane filter (Millipore). The filters were mounted on a flat rigid support with double-sided adhesive tape and then placed in a *n*-hexadecane-filled glass bath. A 20 µl distilled water droplet was allowed to fall on the cell surface. The three-phase contact angle between the baseline of a distilled water droplet on the bacterial lawn suspended in the oil and the tangent at the drop boundary was measured directly with the aid of a microscope containing a goniometric eyepiece (Drop Shape Analysis System G10/DSA10, Krüss, USA). Figure 3.1 represents a photograph of a typical sessile drop as seen on the screen.

In our approach to measuring contact angle, we tried to approximate the *in vivo* hydrocarbon degradation conditions by suspending the cells in an *n*-hexadecane environment and then letting a drop of distilled water to fall on top of the cells. The advantage of this method is that the cells do not have to be dried and therefore no changes in the physiological properties of the cell surface occur (Krekeler *et al.*, 1989). Since evaporation of water was expected to influence contact angle measurements, the mounted filters were left to dehydrate in a biosafety cabinet, at room temperature, and contact angles were measured on replicate filters as a function of time (Absolom *et al.*, 1983). The bacterial lawns were left to dry at room temperature (not in a oven) to avoid excessive drying which could lead to collapse of hydrophobic structures on the cell surface and hence erratic contact angle values (van der Mei *et al.*, 1991a.). Contact

Figure 3.1: Photograph of a distilled water droplet deposited on a bacterial layer prepared for contact angle measurements



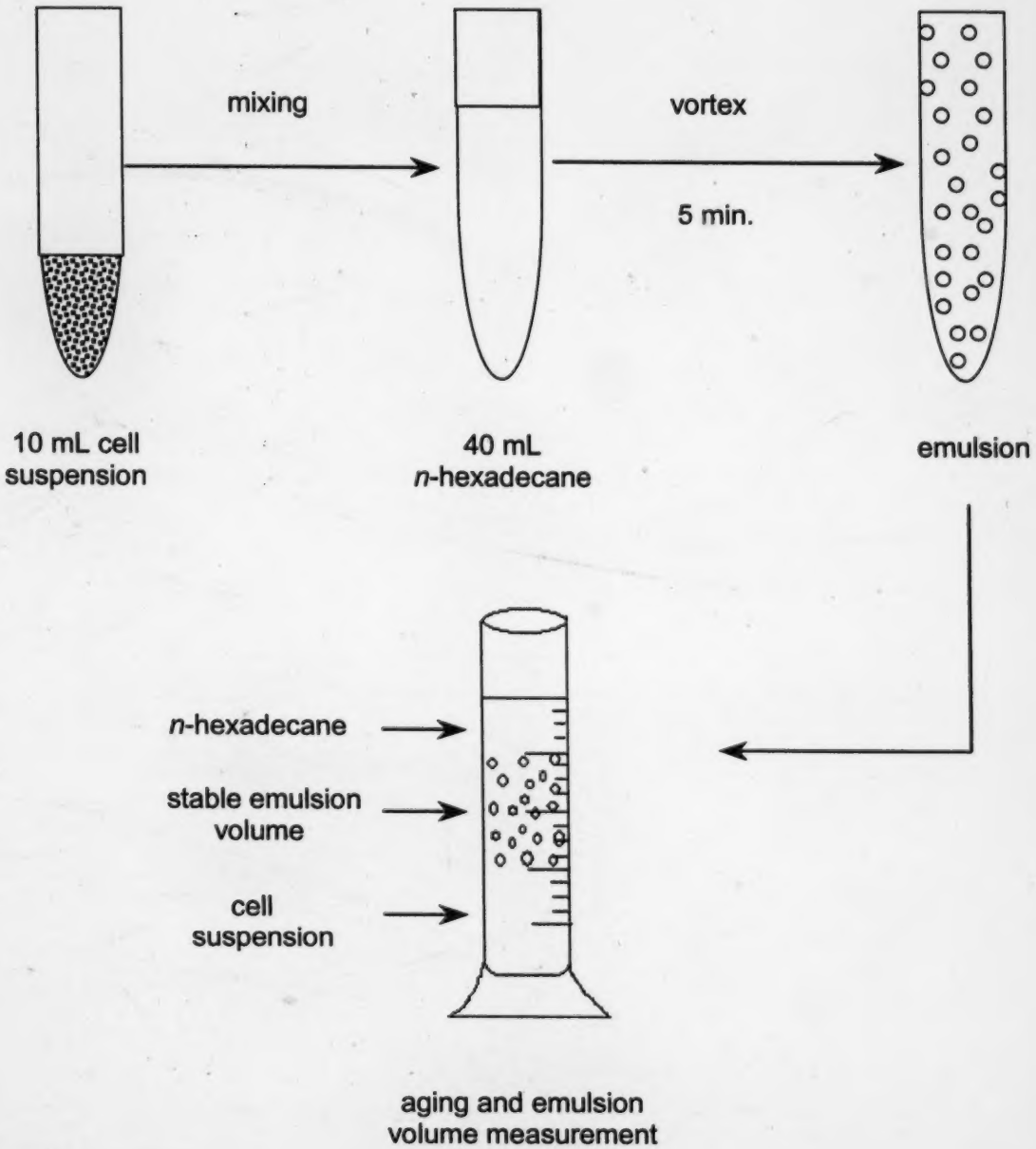
angles changed as the filter cake dried until the level of dehydration allowed the angle at the liquid-liquid interface to remain stable. Each reported contact angle value is the mean of at least 5 independent measurements and resulted in a standard deviation about the mean of approximately 5°.

3.2.3 Emulsion Preparation

The emulsifying ability of different bacterial strains was measured by combining 10 mL of cell suspensions in phosphate buffer (pH 7) with 40 mL of *n*-hexadecane in 20-mm-diameter test tubes. The cell density of bacteria in buffer was adjusted to 5 kg (dry weight) m^{-3} identical to the experiments of Yan *et al.* (2001) with silica particles. The dry weight of cells employed throughout this study was determined in preliminary experiments (see appendix A). In order to obtain a concentration of 5 kg m^{-3}, we employed 104.2 mL of *Acinetobacter venetianus* RAG 1 suspension with OD_{600}=1.02, 84.7 mL of *Pseudomonas fluorescens* LP6a suspension with OD_{600}=1.24, 101 mL of *Rhizomonas suberifaciens* EB2-1a suspension with OD_{600}=1.01, and 135 mL of *Rhodococcus erythropolis* 20S-E1-c suspension with OD_{600}=1.03.

All the mixtures were vortexed for 5 min at maximum rpm, transferred to graduated cylinders and then the height of the emulsified layer was recorded after aging the emulsions for 24 hours. The mixing had to be conducted under mild conditions to ensure that no breakage of the cells occurred. Consequently, to make sure that the contact between the cells and *n*-hexadecane did not result in capsule removal, we performed a capsule staining by Gin's Method (Pierce and Leboffe, 1999) and then observed the cells under an optical microscope. A schematic of the process of preparing emulsions is presented in Figure 3.2.

Figure 3.2: Schematic of the process of preparing emulsions and measuring stable emulsion volumes



3.2.4 Micropipette Technique

Originally developed in the field of biophysics for studying blood cells membranes, this technique has recently been adapted for applications in other areas of colloid science (Yeung *et al.*, 2000; Moran *et al.*, 1999). The micropipette technique gives the ability to manipulate micron-sized particles, in our case bacteria, under *in situ* emulsion conditions, allowing us to test the stability of a 'two-droplet system' and to measure the interfacial tension.

The micropipette system consists of an inverted microscope (Axiovert 100, Carl Zeiss, Canada) fitted with pneumatically controlled micromanipulators, and connected to a video system (CCD camera, video cassette recorder and TV monitor) for monitoring, in real time, the micropipette experiments. All sequences were also recorded on tape for subsequent analyses. Basic aspects of the micropipette setup are shown in Figure 3.3.

Before the initiation of the experiment, glass micropipettes were made from 1 mm o.d., 0.7 mm i.d. glass capillary tubes (Kimble Glass Inc.). By use of a hot wire pipette puller (David Kopf Instruments, Tujunga, CA), the capillaries were stretched axially at high temperature, resulting in tapered hollow tubes whose end dimensions were on the sub-micrometer scale. Next, with the use of a home-made forging device, the tapered ends were truncated to produce pipette tips that have inside diameters of about 10-15 μm. Then, a micropipette was inserted into a hydraulically controlled micromanipulator (Narishige, Tokyo), which enabled motion on the micrometer scale, and allowed the position of the micropipette to be precisely controlled. For pressure control, the other end of the micropipette (the large, untapered end) was connected to a syringe through flexible tubing. The syringe made the connection between the

micropipette and a pressure transducer (e.g., Omega Engineering, Stamford, CT). A photograph of the micropipette apparatus is shown in Figure 3.4.

For performing the interfacial tension measurements, the micropipette tip was filled with *n*-hexadecane and then dipped into a small sample cell containing different bacterial cell suspensions (or water or phosphate buffer as controls). The pressure required to push the oil droplet out of the micropipette was recorded as a direct measurement of interfacial tension. Further, from the knowledge of pressure and micropipette radius, we were able to determine the interfacial tension by using the Young-Laplace equation (equation 2.2).

Figure 3.3: Schematic of micropipette apparatus (Yeung *et al.*, 2000)

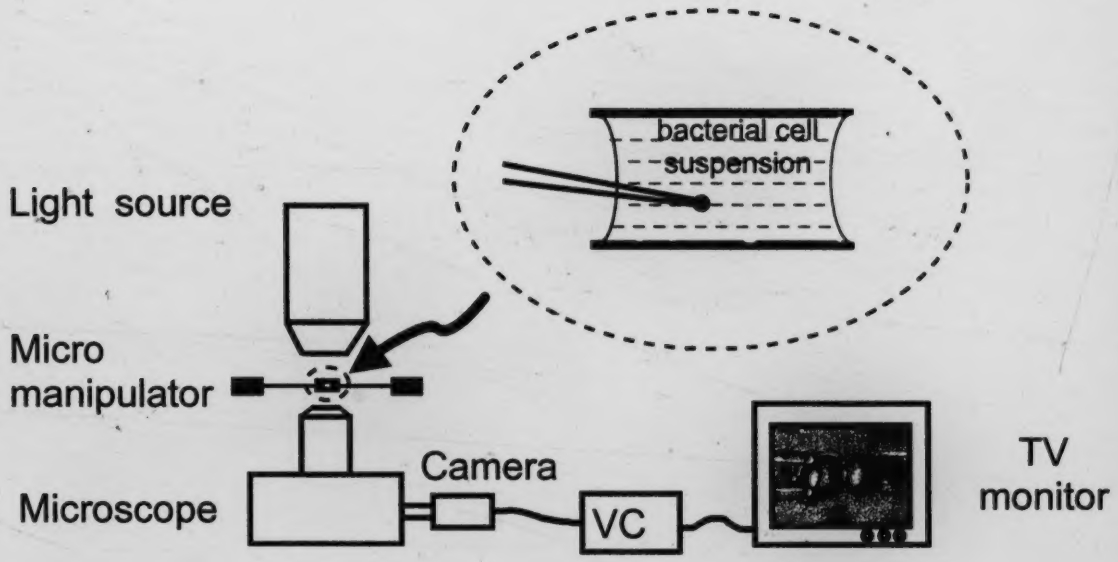
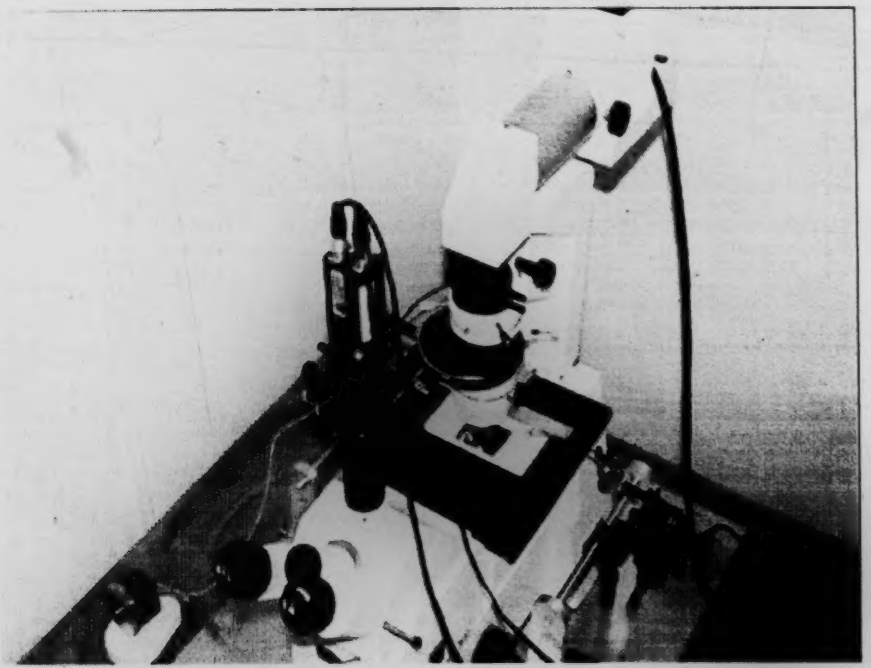


Figure 3.4: Photograph of micropipette apparatus



IV RESULTS

The hypothesis underlying this research was that intact bacterial cells can stabilize emulsions by the same mechanism as finely divided solid particles, namely by providing a steric barrier against droplet coalescence. The knowledge of the relationship between silica particle surface hydrophobicity (measured by contact angle) and its ability to stabilize oil-water emulsions, led us to the hypothesis that a similar relationship should exist between bacterial surface hydrophobicity and their ability to stabilize oil-water emulsions.

4.1 MICROBIOLOGICAL CHARACTERISTICS

The bacterial cultures used in this study were harvested in the late stationary growth phase as presented in Table 4.1. *A. venetianus* RAG 1 was harvested after 18 hours of growth, *P. fluorescens* LP6a after 48 hours of growth, *R. suberifaciens* EB2-1a and *R. erythropolis* 20S-E1-c after 50 hours of growth. The growth curves for all the microbial strains employed throughout this study are presented in Appendix A (Figures A-1 to A-4) along with the correlation between OD_{600} and dry weight, Appendix A, (Figures A-4 to A-8). The conversion factors from OD_{600} to dry weight had a value of 0.9964 for *A. venetianus* RAG 1, 0.995 for *R. erythropolis* 20S-E1-c, and 0.997 for *R. suberifaciens* EB2-1a and *P. fluorescens* LP6a.

Staining the cells revealed the fact that all bacterial species used throughout this study possess capsules as shown in the following photographs, Figures 4.1 to 4.5, but of the four strains, the most pronounced capsule was observed with *R. suberifaciens* EB2-1a.

Table 4.1: Harvest time for different bacterial strains

Bacterial Strain	Harvest Time (h)
Acinetobacter venetianus RAG1	18
Pseudomonas fluorescens LP6a	48
Rhizomonas suberifaciens EB2-1a	50
Rhodococcus erythropolis 20S-E1-c	50

Figure 4-1: Micrograph of capsule *Rhizomonas suberifaciens* EB2-1a

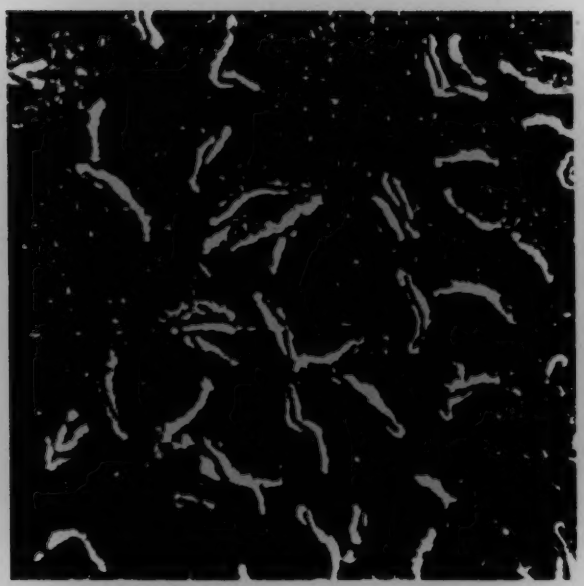


Figure 4.2: Micrograph of *Pseudomonas fluorescens* LP6a capsule

Figure 4.3: Micrograph of *Acinetobacter venetianus* RAG-1 capsule

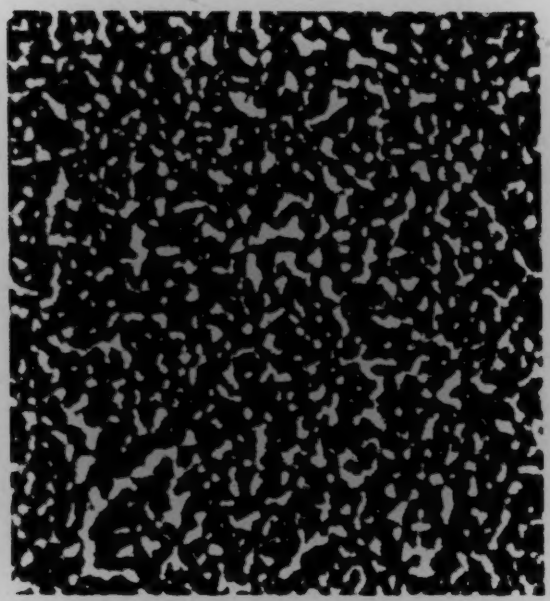
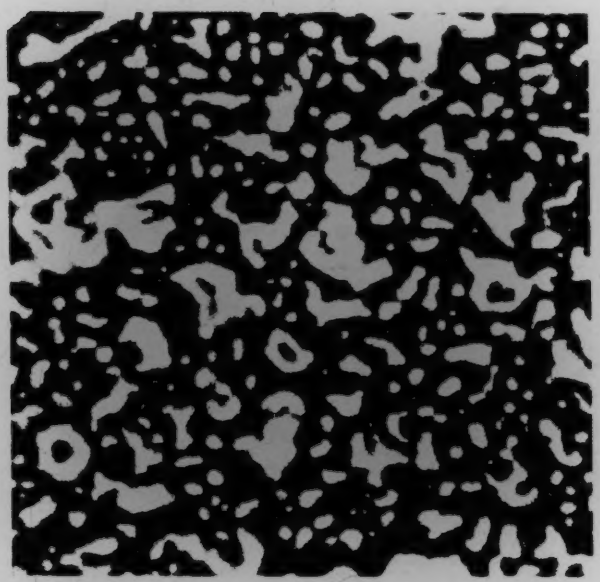


Figure 4.4: Micrograph *of Rhodococcus erythropolis* 20S-E1-c capsule



4.2 SURFACE AND INTERFACIAL TENSION MEASUREMENTS OF THE CELL-FREE SPENT MEDIUM

The results of surface tension measurements for distilled water, TSB fresh medium and cell-free TSB spent medium of *Acinetobacter venetianus* RAG 1, after 18 h of incubation, are shown in Figure 4.5. The decrease in surface tension for the cell-free spent medium could be due to the production of some extracellular compounds which are surface active, but as reported by Cooper (1986) the level of biosurfactant produced is promising only if it reduces the surface tension of a liquid medium to 40 mN/m or less. The definition of this threshold level was adapted from the literature. Willumsen and Karlson (1997) also reported that a good biosurfactant should be able to reduce the surface tension of the growth medium by >20 mN/m compared to distilled water.

The results of interfacial tension measurements between distilled water, TSB fresh medium, cell-free TSB spent medium of *Acinetobacter venetianus* RAG 1, after 18 h of incubation, and *n*-hexadecane are shown in Figure 4.6. The decrease in interfacial tension, illustrated in this graph, does not indicate significant biosurfactant production. Cell components, such as lipoproteins, could explain such a decrease in interfacial tension.

Figure 4.5: Surface Tension of Fresh and Spent Growth Media of *Acinetobacter venetianus* RAG 1

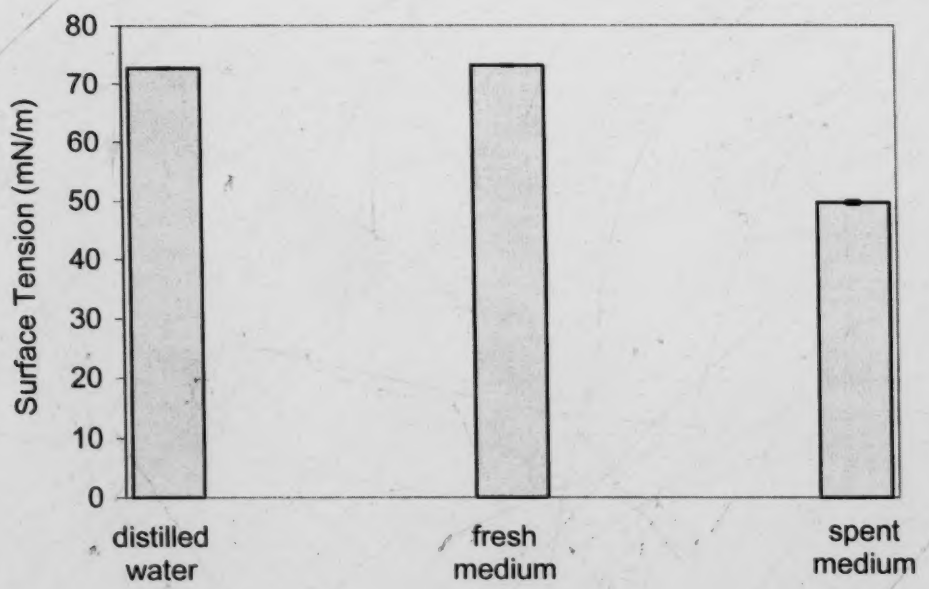
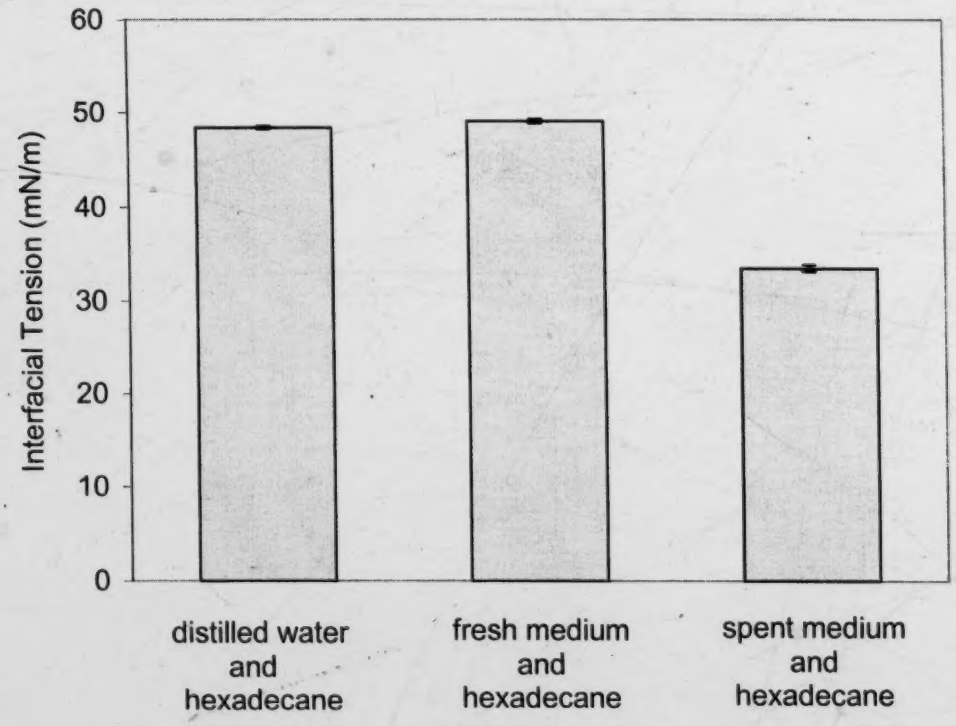


Figure 4.6: Interfacial Tension of Fresh and Spent Growth Media of *Acinetobacter venetianus* RAG 1



4.3 HYDROPHOBICITY MEASURED THROUGH DIFFERENT METHODS

There is still debate in the literature about the right method that should be used in measuring cell surface hydrophobicity. Even though contact angle is an important technique in determining cell surface hydrophobicity, and its use may indeed provide highly relevant data for quantitation, some authors consider that employing two independent techniques for measuring hydrophobicity may lead to more reliable data.

4.3.1 Hydrophobicity Measured by BATH

It has been previously reported that cell-surface hydrophobicity varies with the growth substrate and the growth phase (Bredholt *et al.*, 2002, Krekeler *et al.*, 1989), therefore, we grew all the cells using the same soluble substrate (TSB) and also harvested all of them in the late stationary phase (as determined in preliminary studies). Buchs *et al.* (1988) modified the surface characteristics of *Corynebacterium glutamicum* by changing the culture conditions, i.e. the phosphate concentration of the medium. Cells grown under conditions of phosphate saturation were more hydrophobic than those grown under phosphate limitation. Whyte *et al.* (1999) reported increased hydrophobicity of *Rhodococcus* sp. strain Q15 when they were grown on diesel fuel compared to when they were grown on glucose-acetate.

The adherence of the selected bacterial strains is presented in Table 4.2. These results demonstrate the low adherence of *P. fluorescens* LP6a towards *n*-hexadecane, greater contact with *n*-hexadecane for *R. suberifaciens* EB2-1a, and very high adherence for *R. erythropolis* 20S-E1-c and *A. venetianus* RAG 1. *P. fluorescens*

Table 4.2: Adherence to *n*-hexadecane of four bacterial strains (± CI at 95% of n=3 replicates)

Bacterial strain	Adherence to *n*-hexadecane phase %
Acinetobacter venetianus RAG1	95.7 ± 1.8
Rhodococcus erythropolis 20S-E1-c	80.3 ± 1.5
Rhizomonas suberifaciens EB2-1a	55.0 ± 2.4
Pseudomonas fluorescens LP6a	18.3 ± 4.3

LP6a showed almost no affinity for hexadecane. The hydrocarbon droplets rose and coalesced soon after mixing and no significant changes were observed in the turbidity of the lower aqueous phase, *A. venetianus* RAG 1, which is known to adhere avidly to *n*-hexadecane (Rosenberg *et al.*, 1980), formed very stable emulsions. The extraction of cells into the emulsion layer was accompanied by a corresponding decrease in turbidity of the lower aqueous phase. The cell-coated hydrocarbon droplets in the emulsion layer remained stable for a long period of time. We can notice from this experiment that even when using a soluble growth medium, certain bacteria can develop very high hydrophobicity and, therefore, a hydrocarbon substrate to induce hydrophobicity is not always required.

4.3.2 Hydrophobicity Measured by Contact Angle

As a first step in our experimental approach, contact angles were measured starting immediately after filtration, without drying the cells. Then, in order to determine the variation with time, the cells were allowed to dry at room temperature and contact angle measurements were followed with time for each bacterial strain. Contact angles were relatively independent of drying time except for *P. fluorescens*, which gave a continuous increase, starting with a 0° contact angle immediately after filtration up to a value of 39.7° after 24 hours of drying (Table 4.3). The same ranking of the contact angle among the four bacterial strains was observed whether the values were recorded immediately after filtration or 24 h later.

Table 4.3: Contact angle variation with "drying time" for the four bacterial films. Data are mean ± SD of n=3 replicates

Bacterial Strain	Contact angle (°)			
	0 h	1 h	3 h	24 h
Acinetobacter venetianus RAG1	37.3 ± 0.5	38.9 ± 0.3	43.2 ±1.4	56.4 ± 2.4
Pseudomonas fluorescens LP6a	0	0	0	39.7 ± 4.8
Rhizomonas suberifaciens EB2-1a	40.1 ± 0.2	42.3 ± 0.7	45.7 ± 0.4	50.3 ± 3.8
Rhodococcus erythropolis 20S-E1-c	142.5 ± 0.3	142.8 ± 3	144.0 ± 1.1	152.9 ± 1.3

Table 4.3 lists contact angle values after 24 hours of drying at room temperature, with range of 40° to 153°. The most hydrophobic surface seemed to be that of *R. erythropolis* 20S-E1-c with a contact angle value of 152.9°; in this case, the water droplet tended to roll down the surface without spreading. On the surfaces of *A. venetianus* RAG 1 and *R. suberifaciens* EB2-1a, the water droplet behaved almost identically, spreading quickly, with the resulting contact angles of 56.4° and 50.3°, respectively. The hydrophilic surface of *P. fluorescens* LP6a was emphasized by the low value of 39.7°. A similar range of contact angles was observed by van der Mei (1991 b.) for *A. venetianus* depending on the growth medium.

Following the reports of Neufeld *et al.* (1980), contact angle values for cells metabolizing hydrocarbons compared to those metabolizing soluble substrates should be very different with a mean of 20.7° for the soluble-growing substrate cells and 120° for hexadecane growing cells. From our experiments, we noticed that even when grown with a soluble carbon source only, bacteria can develop contact angles as high as 153°.

4.3.3 Micropipette Results

As shown by Yeung *et al.* (2000), the micropipette technique gives promising results in determining interfacial properties of the individual emulsion droplets stabilized by fine solids. As a result of the successful application of this technique on solids and due to the lack of research in the area of interfacial properties of the bacterial stabilized emulsions, we investigated its application to microbial cells at the interface between water and *n*-hexadecane.

The validity of the micropipette technique can be established by measuring the interfacial tensions between water and several nonpolar liquids (Yeung, 2001). As no surfactants are involved, these interfacial tension measurements must agree with accepted literature values for pure liquids. In our case, all the measured interfacial tension values were in the range of the literature values (Table 4.4) (Lee *et al.*, 2001), therefore, we proceeded with the measurements involving the three phase system: hexadecane, phosphate buffer and bacteria. When the interfacial tension was measured immediately after bringing into contact *n*-hexadecane and hydrophobic bacterial strains, no decrease was observed. The measured values were equivalent to those with the phosphate buffer and hexadecane (Table 4.4). Equilibrium measurements taken after allowing the cells to assemble at the water/oil interface for 5 min were slightly lower, but still too high to account for any stabilization of emulsion (Table 4.4). This observation showed that cells stabilized emulsions without significantly changing the interfacial surface tension, consistent with the behavior of fine particles (Levine *et al.*, 1989).

During a qualitative experiment consisting of pushing together two hexadecane droplets stabilized by hydrophobic bacteria, Figure 4.7 (A and B), I noticed that despite attempts to induce coalescence through forced contact for up to a few minutes, the droplets remained separate entities. This result shows that bacterial cells are able to create an adsorbed film at the oil-water interface capable of preventing coalescence.

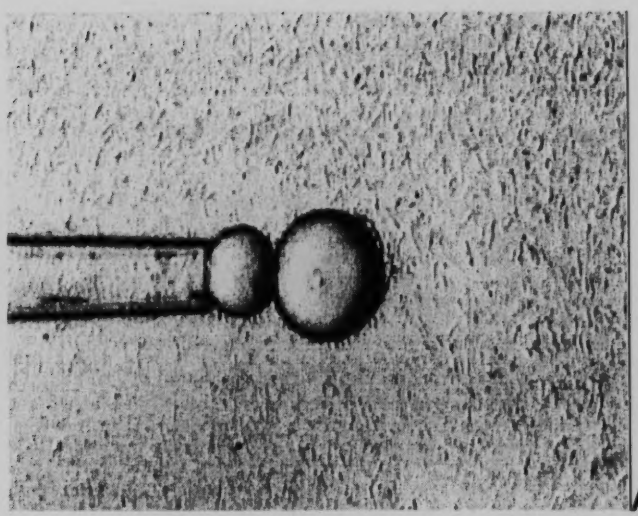
To further demonstrate that the bacteria at the interface interact with each other to form a surface film, I extruded a hexadecane droplet into suspensions of *A. venetianus* RAG-1 and *R. erythropolis* 20S-E-1c (Figure 4.8), then after a few minutes I slowly withdrew the droplet, reducing the interfacial area. Example images in Figures 4.8

Table 4.4. Interfacial tension and equilibrated interfacial tension measured with the micropipette technique (mean ± SD of n=3 replicates)

Mixture	Interfacial Tension mN/m	Equilibrated Interfacial Tension mN/m
Water + nC16	48.7 ± 1.2	NA[1)]
Buffer + nC16	46.6 ± 2.1	NA
LP6a	46.1 ± 1.1	NA
EB2-1a	46.0 ± 1.1	36.3 ± 1.1
RAG1	46.3 ± 1.0	36.1 ± 1.3
20S-E1-c	46.0 ± 1.5	33.6 ± 1.1

[1)] Not applicable – Droplets of *n*-hexadecane were not stable in suspensions of *P. fluorescens* LP6a or in pure water

Figure 4.7: Droplets of *n*-C16 in suspension of *A. venetianus* RAG1 stabilized against coalescence by bacteria at the oil/water interface (pipette tip has a diameter of 14 µm). A. and B. represent different contact times



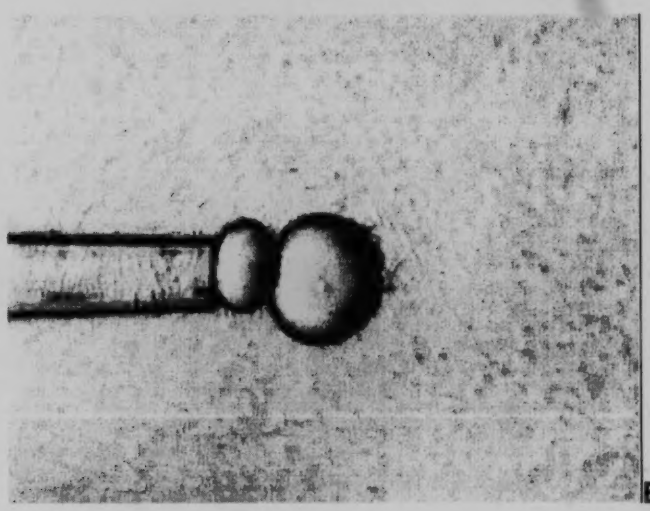
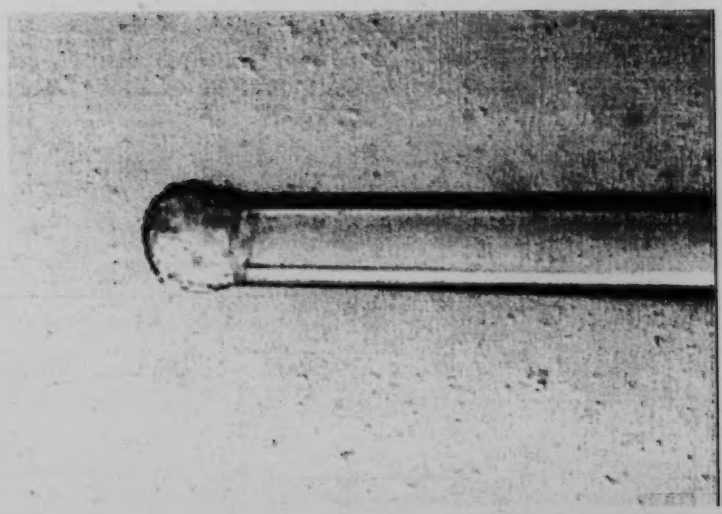
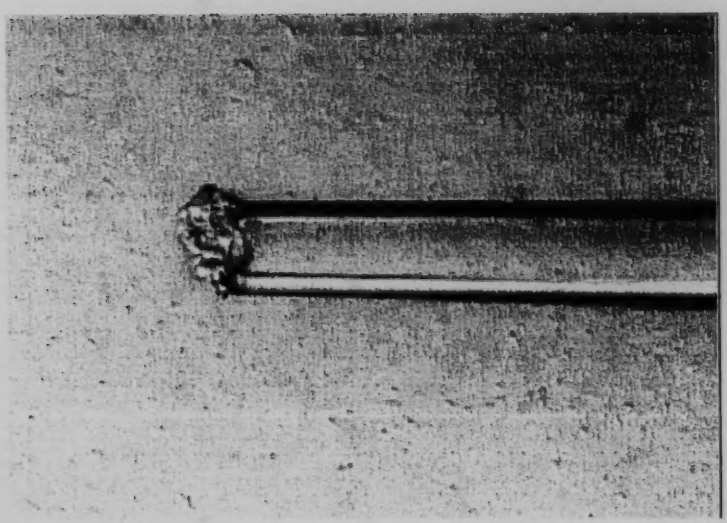


Figure 4.8: Deformation of the oil/water interface caused by *R. erythropolis* 20S-E1-c adhering tightly at the interface when hexadecane droplet is extruded into the cell suspension then withdrawn. A. After partial withdrawal of *n*-C_{16} droplet. B. After complete withdrawal of *n*-C_{16} droplet



A.



B.

A and 4.8 B clearly show a rigid film at the hydrocarbon-water interface due to the presence of bacteria that resisted the change in surface area to form a crumpled surface. The first photograph, 4.8 A, was recorded after initial withdrawal of the oil droplet while the second, 4.8 B, was recorded after complete withdrawal. The same phenomena have been observed with hydrophobic clay particles and silica beads at an oil/water interface (Chiang and Elliott, 2002; Yeung *et al.*, 2000; Dabros *et al.*, 1999). When the same experiment was repeated with the hydrophilic *P. fluorescens* LP6a, no such distortion of the droplet surface was observed. By contrast, the shape of the deflating droplet covered by hydrophilic bacteria remained spherical as it shrank.

This experiment depicted not only the strong attachment of hydrophobic bacteria to the oil-water interface, but also their ability to interact with each other and form a surface film that resisted coalescence and deformation.

4.4 EMULSIONS STABILIZED BY BACTERIA

The stability of these cell-stabilized emulsions was followed with time after the addition of the cells and formation of the emulsion. After 24 hours of settling, the height of the emulsified layer was found to be in the range 0-80 volume %, as a fraction of mixture total volume. The highest volume of emulsion, 80%, was obtained with *A. venetianus* RAG-1, followed by *R. erythropolis* 20S-E1-c with a volume of 16%. In contrast, *P. fluorescens* LP6a and *Rhizomonas suberifaciens* EB2-1a did not yield stable emulsions, nor did water or cell-free medium from any strain. Therefore, the stable emulsions observed with RAG-1 and 20S-E1c were achieved by the cells in the absence of surfactants or emulsifiers. To confirm that intact cells, rather than any emulsifier or surfactant released by them, were responsible for the formation of emulsions, we

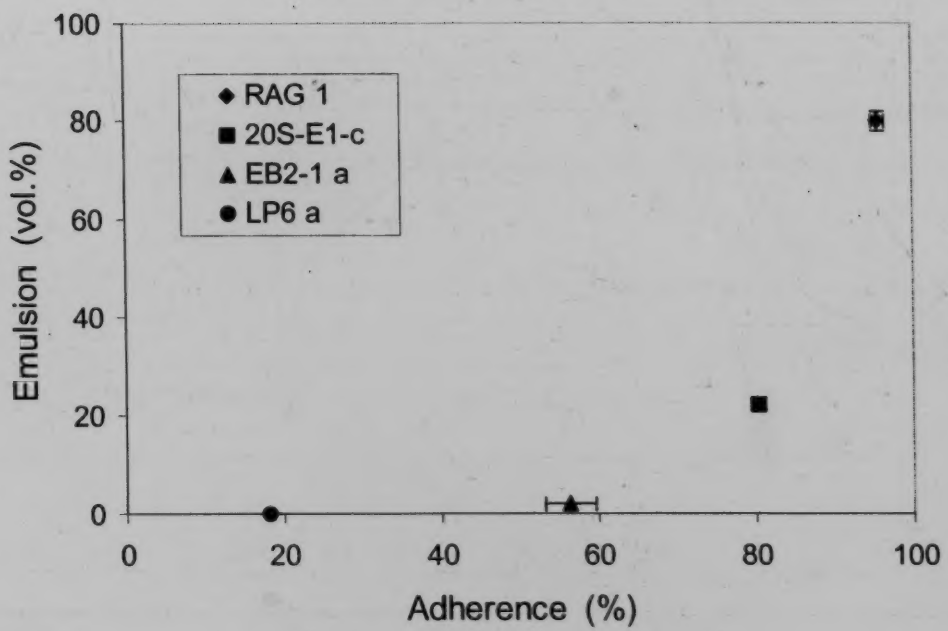
separately vortexed cell suspensions of each strain for five minutes without *n*-hexadecane added, and then removed the cells by centrifugation. The resulting cell-free supernatant was then used in preparing emulsions following the above-mentioned protocol. No emulsion was obtained for any of the strains, suggesting that bacterial cells themselves were responsible for the formation and stabilization of emulsions.

Cell emulsions formed by RAG-1 were stable up to several months whereas a less stable emulsion was noticed in the case of 20S-E1-c, where after 24 hours of aging, the volume decreased by 30%.

4.4.1 Emulsion Formation versus BATH Adherence

The data for TSB-grown cells to stabilize hexadecane-water emulsions is plotted against adherence determined by BATH test in Figure 4.9. The data in the graph represent the percentage of volume emulsified after 24-hour emulsion aging. We noticed a non-linear correlation between adhesion and emulsified oil volume. The largest emulsion volume was obtained with *A. venetianus* RAG-1, the most adherent bacterium.

Figure 4.9: Variation of emulsion volume with adherence for the four bacterial strains. Symbols are the means with error bars representing standard deviation of n=3 replicates. The full names of the strains are given in Table 4.1



4.4.2 Emulsion Formation versus Contact Angle

Due to the fact that BATH gives hydrophobicity in terms of adhesion, we used contact angle to measure the hydrophobic properties of the outer cell surface as a whole.

Figure 4.10 represents the relationship between the stable emulsion volume and contact angle. A maximum was observed for *A. venetianus* RAG-1, which had an intermediate contact angle, followed by a decrease in emulsion volume for *R. erythropolis* 20S-E1-c which had the largest contact angle. The lowest contact angle bacterium *P. fluorescens* formed no emulsion at all, where all the cells resided in the water phase. The slightly more hydrophobic bacterium, *R. suberifaciens*, formed a very small volume of emulsion that coalesced immediately after mixing. Notably, the same trend was observed for fine silica spheres (diameter 12 nm) of different hydrophobicities (Yan *et al.*, 2001).

Figure 4.11 shows comparatively the behavior of highly hydrophobic bacteria and silica particles in forming and stabilizing oil-water emulsions. In addition to the previous graph which shows the maximum emulsion volume for intermediate contact angle bacteria and silica, Figure 4.11 reveals another important similarity between the highest hydrophobicity bacteria and silica particles; both of them have the tendency to migrate into the oil phase, presumably due to high surface hydrophobicity. For all the other samples, the oil phase appeared clear after 24 hours of settling. This observation was also underlined by Watkinson *et al.* (1990), who noticed that many hydrocarbon-degrading microorganisms that have highly hydrophobic cell surfaces may frequently

Figure 4.10: Comparison between emulsion volume and contact angle of the four bacterial strains and silica particles of different hydrophobicity. The full names of the strains are given in Table 4.1. Data on solids are from Yan *et al.* (2001)

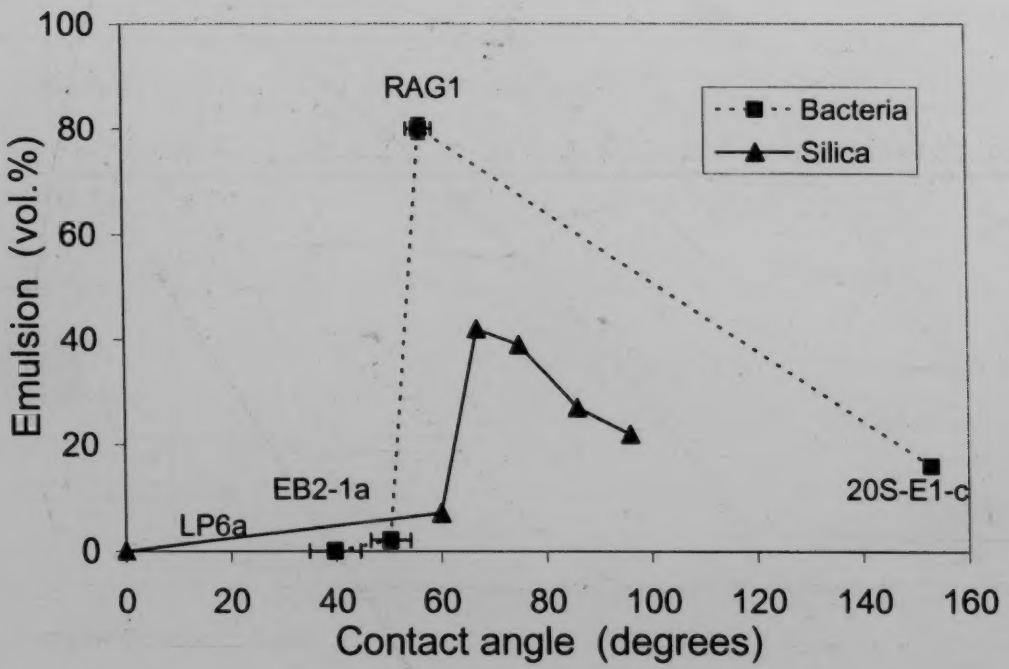
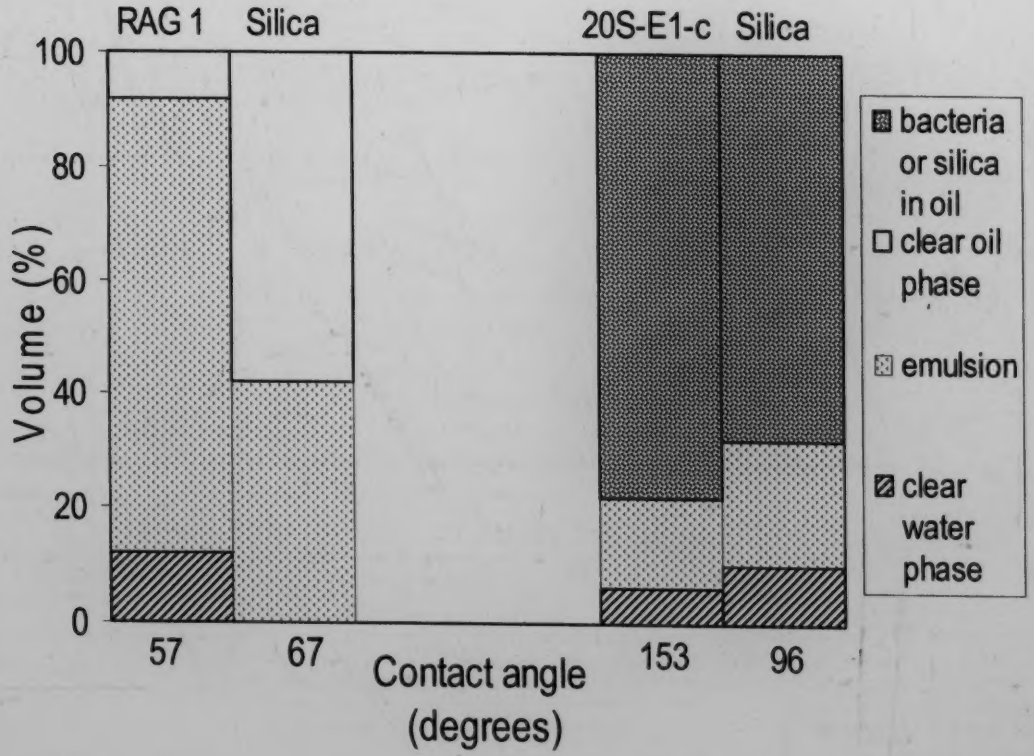


Figure 4.11: Preparation of emulsions stabilized by bacteria with intermediate and highest contact angle values. Comparison to silica particles with the same range of hydrophobicity (solids concentration: 5 kg m^{-3}, bacteria concentration: 5 kg m^{-3}). Data on solids are from Yan *et al.* (2001)



associate with hydrocarbon droplets and even pass into the organic phase during growth. Reed and Rice (1931) demonstrated quantitatively that acid-fast bacteria namely, *Mycobacterium* sp., are able to pass from the aqueous into the bulk oil phase. There are also reports of fungi, and an occasional bacterium, being recovered from hydrocarbon fuels that were essentially free of water (DeGray and Killian, 1962). These microorganisms persisted for at least six months in essentially anhydrous diesel fuel.

4.4.3 Emulsion Microscopy

In order to establish the type of the emulsion formed by the two most hydrophobic bacteria, we examined them under a confocal microscope (Leica TCS-SP2 Multiphoton CLSM). Prior to mixing bacterial suspensions with *n*-hexadecane, the cell suspensions were labelled with two droplets of a Na-fluorescein (0.1%) solution; the *n*-hexadecane remained uncoloured.

R. erythropolis 20S-E1-c formed both oil-in-water and water-in-oil emulsions in different layers, (Figures 4.12 and 4.13). The middle layer (i.e. the stable emulsion) comprised an oil-in-water emulsion (Figure 4.12), while the oily phase (top layer) revealed water-in-oil emulsions featuring assemblies of cells surrounded by thin water films (Figure 4.13). The latter behavior was consistent with observations that highly hydrophobic silica and bacteria can pass into the organic phase (Yan *et al.*, 2001; Watkinson and Morgan, 1990). Such cells may be hydrophobic enough to initially adhere at the hydrocarbon-water interface but lack the cooperative cell-cell interactions necessary to form a stable cream of cell-covered droplets.

The emulsion formed by *A. venetianus* RAG-1 revealed an unexpected structure of dark oil droplets surrounded by bacterial films interconnected in a three-

dimensional network (Figure 4.14), analogous to liquid films around gas bubbles in a foam. Due to the high content of the internal phase (hexadecane), the foamlike structure and its behavior as a gel (i.e. showing yield stress), this emulsion was classified as an emulsion gel (Babak and Stébé, 2002). These emulsion gels are usually obtained via multiple emulsions which form during mechanical agitation of the system by breaking a less stable in favour of a more stable emulsion. When such an emulsion is subjected to small shear deformation usually it exhibits a yield stress (Babak and Stébé, 2002); the consequence is that these gels resist flow, because the surface tension opposes the deformation of the oil droplets.

Microscopy of the emulsions formed by *R. suberifaciens* EB2-1a and *P. fluorescens* LP6a was not possible due to their high instability.

Figure 4.12: Oil-in-water emulsion formed by *R. erythropolis* 20S-E1-c, with fluorescent bacteria surrounding hexadecane droplets; cell suspensions were labeled with Na fluorescein

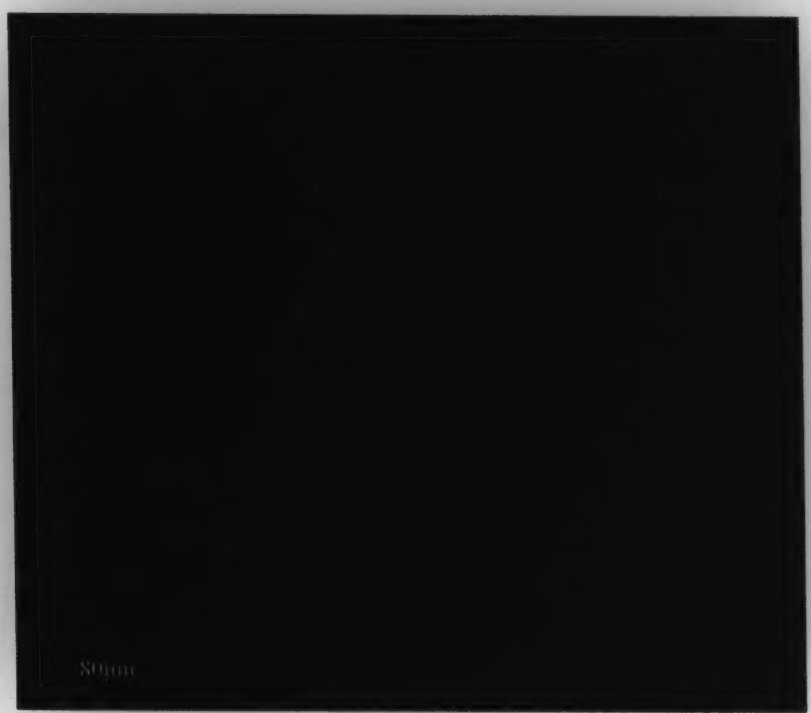


Figure 4.13: Water-in-oil emulsion formed by *R. erythropolis* 20S-E1-c with clusters of bacteria surrounded by a thin film of water (right side)

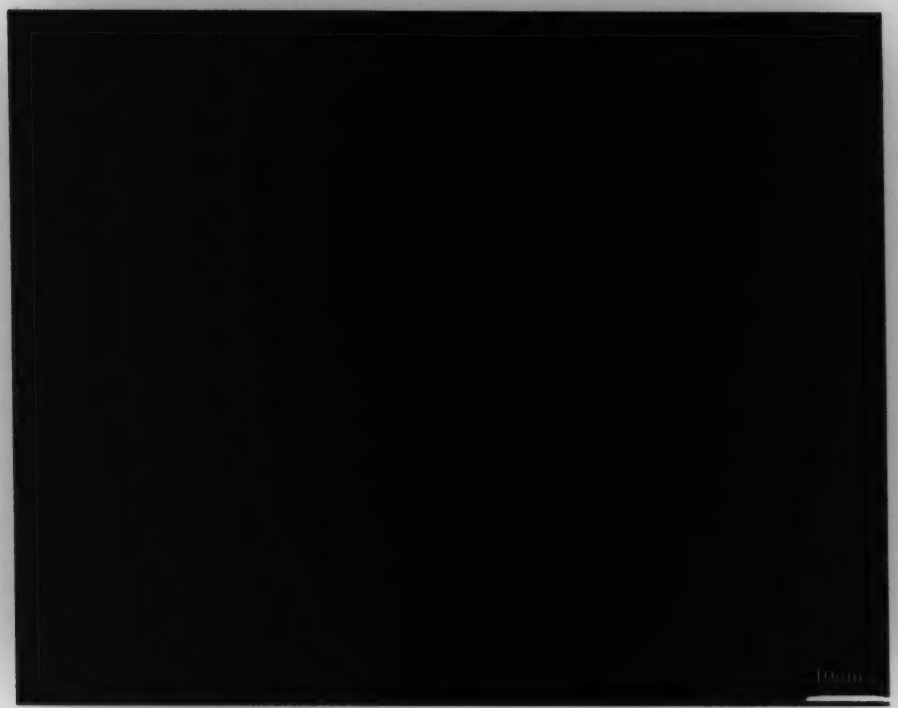
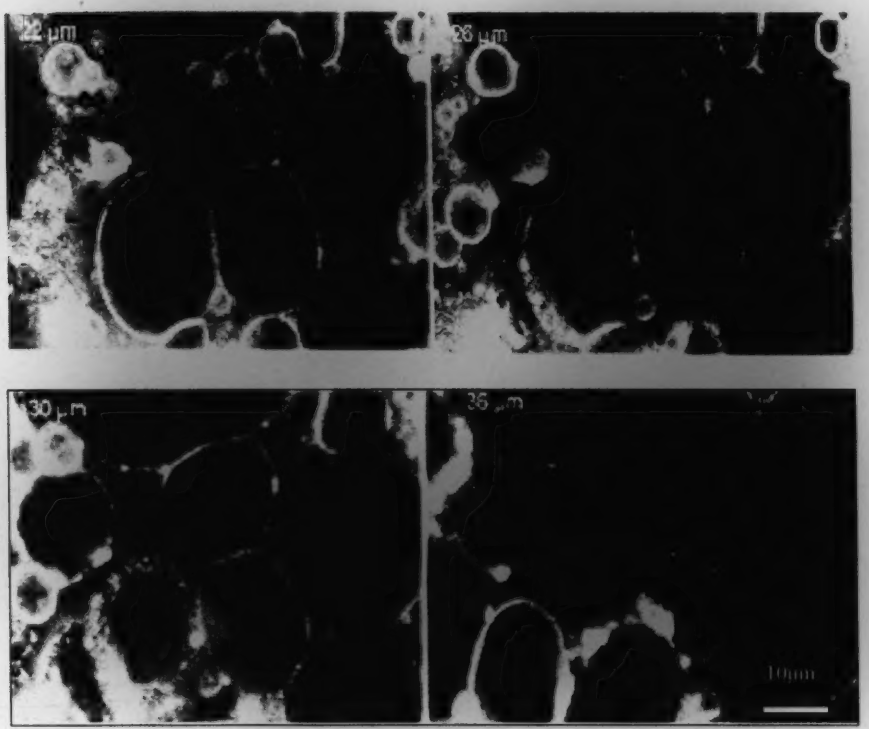


Figure 4.14: Confocal micrograph of the "emulsion gel" formed by mixing *A. venetianus* RAG 1 suspensions with *n*-hexadecane.These images were taken at different depths from the air/emulsion interface



V DISCUSSION

This study showed that intact bacteria alone could stabilize oil/water emulsions by attaching to the interface, analogous to silica particles with contact angles intermediate between water-wet and oil-wet. The attached bacteria hindered the coalescence of oil droplets, and interacted to form surface films and emulsion gels.

5.1 MICROBIAL CELL SURFACE HYDROPHOBICITY

The observed ability of different bacterial species to form emulsions was in qualitative agreement with their contact angles and followed the same trend as silica particles, suggesting that contact angles are a useful tool in studying cellular interactions at interfaces. While hydrophobic cells stabilized oil/water emulsions for a long time, hydrophilic bacteria showed no adherence to oil droplets and as a result, they were not able to stabilize emulsions. Consequently, the most stable emulsions were formed by bacteria with intermediate hydrophobicity which is in accordance with the findings regarding solid particles (Yan et al., 2001). The emulsion types observed, from oil-in-water emulsions with bacteria displaying intermediate contact angle to water-in-oil emulsions with bacteria having high contact angles, were equivalent to silica particles over the same range of contact angles (Yan *et al.*, 2001).

5.2 MECHANISM OF EMULSION STABILIZATION

This study showed that intact hydrophobic bacterial cells could form and stabilize emulsions analogous to finely divided solids by dispersing oil into droplets and providing a surface skin, which acts as a barrier against coalescence. The major mechanism responsible for stabilizing emulsions by both hydrophobic bacteria and silica is steric hindrance of coalescence. It is evident that hydrophobic bacteria possess a high affinity for the oil/water interface. This phenomenon was remarkably emphasized during the micropipette experiments, when despite attempts to induce coalescence of two oil droplets stabilized by hydrophobic bacteria, the droplets remained separated entities.

In addition to the strong adhesion to the oil-water interface, hydrophobic bacteria possess an affinity to each other, which leads to the self-assembly of bacteria at the oil-water interface. The observation of self-assembly of bacteria at the oil-water interface, namely the surface skin that resists deformation, was remarkably emphasized for both *A. venetianus* RAG1 and *R. erythropolis* 20SE1-c during the micropipette experiment. The surface skin became evident as an oil droplet was extruded into a hydrophobic cell suspension and then withdrawn back into the micropipette.

It is evident that the surface skin formed by hydrophobic bacteria due to high affinity to each other and the strong adherence between the cells and oil droplets is responsible for the *A. venetianus* RAG1 emulsion gel structure. Even though strong adherence to oil droplets was noticed in the case of R. *erythropolis* 20SE1-c as well, the migration of some of the cells into the oil phase made this emulsion less stable.

My results suggest that bacterial cells are an important agent in stabilizing emulsions in circumstances ranging from oil spills to fermentation; the addition of hydrophobic cells may be a valuable strategy for emulsifying and dispersing oil spills or enhancing the bioconversion of non-aqueous liquids. The observation of self assembly of bacteria at the interface to form a surface skin that resists deformation may be important for fluid flow through porous media, as in petroleum production and bioremediation of aquifers contaminated by non-aqueous phase liquids.

VI CONCLUSIONS AND RECOMMENDATIONS

The hypotheses underlying this study were that bacterial cells would stabilize oil-water emulsions by the same mechanism as finely divided solids, namely by steric hindrance against coalescence, and that the ability of microbial cells to stabilize emulsions would correlate with cell surface contact angles.

This research project showed a qualitative agreement between the cell surface contact angles and the volume of stabilized emulsions. In the case of *P. fluorescens* LP6a, the bacterium with the lowest contact angle value, 39.7°, no emulsion was formed. For *A. venetianus* RAG 1, an intermediate contact angle bacterium, 56.4°, a maximum volume of emulsion was obtained. Conversely, when the highest contact angle bacterium, 152.9°, was used in stabilizing emulsions, the volume of the stable emulsion was lower compared to the previous case. The trend followed by microbial cells in stabilizing emulsions was identical to that observed for silica particles and was in accordance with their surface contact angle.

The mechanism responsible for emulsion stabilization was steric hindrance of coalescence, analogous to insoluble particles such as silica. This emulsion stabilization mechanism was emphasized by the tendency of hydrophobic bacterial cells to attach to the hexadecane droplets and to withstand coalescence.

In addition to the tendency to adhere very tight to the oil-water interface, hydrophobic microbial showed the ability to adhere very strong to each other producing cell assemblies such as emulsion gels.

6.1 DIRECTIONS FOR FUTURE WORK

This project should be continued in two main directions, namely, measurement of the adhesion force between hydrophobic bacteria at the interface and examination of how cell surface composition affects adhesion to oil-water interface.

One possible way to quantify adhesion force between bacteria at the interface is using atomic force microscopy. The invention of the atomic force microscope in 1986 provided an important tool for the study of microbial cell surfaces (Binnig *et al.*, 1986). Unlike the electron microscope, which must operate in a vacuum, the atomic force microscope can be operated in any environment including liquid media that simulate natural growing conditions. The atomic force microscope thus serves as an ideal tool for the investigation of the cell attachment process since it can be used to measure interaction forces between bacteria and a sample substrate or surface.

It is well known that bacterial adhesion to each other and to an oil-water interface depends on the properties of the cell wall and capsular polymers. Therefore, understanding bacterial adhesion to different substrates requires knowledge of the conformational properties of bacterial surface polymers. There are reports in the literature of cell capsular material extracted and analyzed through different chemical methods, but many times the characterization is not complete. As a result, in order to be able to predict bacterial attachment based on biopolymer properties more detailed characterization of capsular polymers is required in conjunction with atomic force microscopy and contact angle measurements.

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APPENDIX A

Figure A-1: Growth curve for *Pseudomonas fluorescens* LP6a

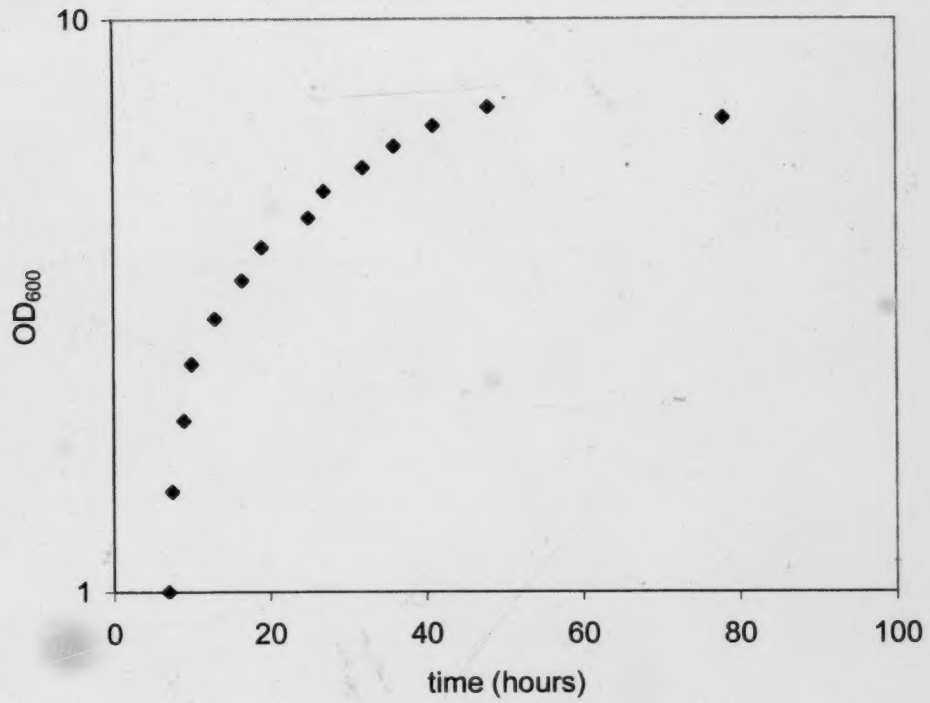

Figure A-2: Growth curve for *Rhodococcus erythropolis* 20SE1-c

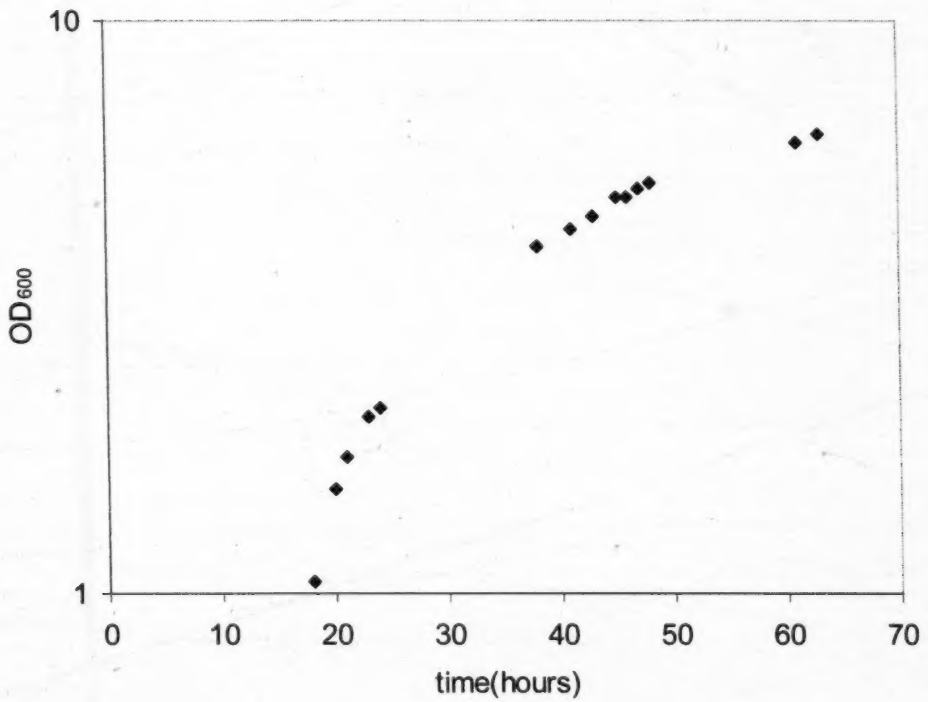

Figure A-3: Growth curve for *Acinetobacter venetianus* RAG-1

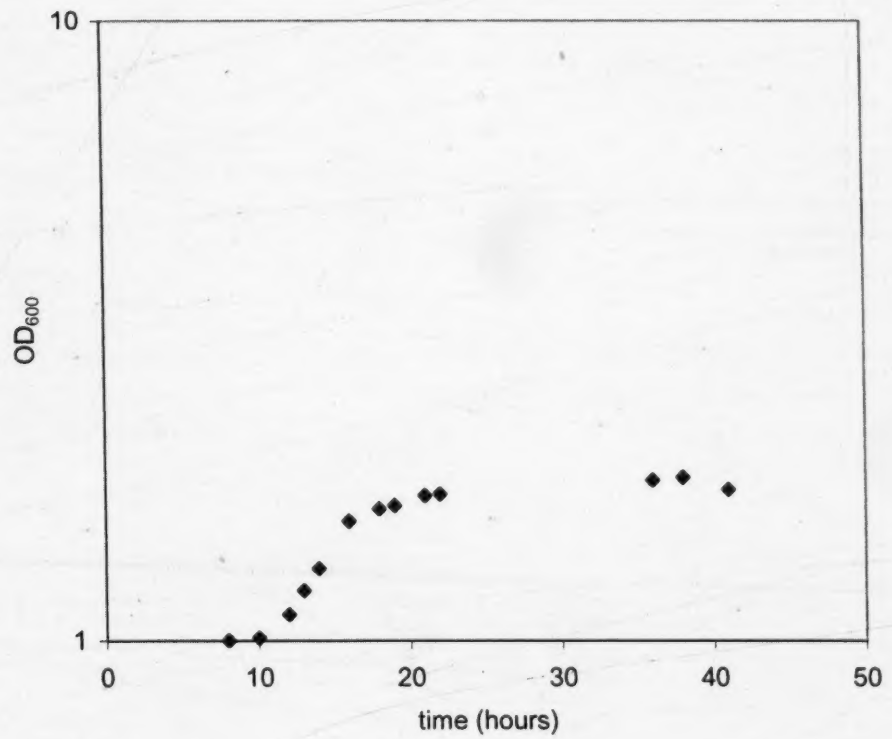

Figure A-4: Growth curve for *Rhizomonas suberifaciens* EB2-1a

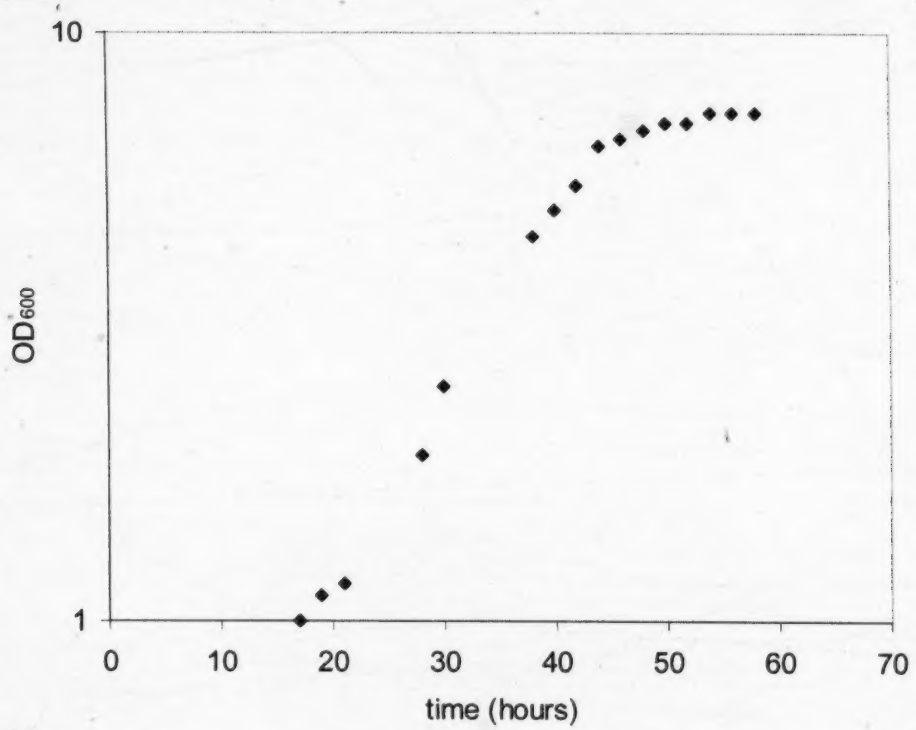

Figure A-5: Dry weight versus OD_{600} *for Acinetobacter venetianus* RAG-1

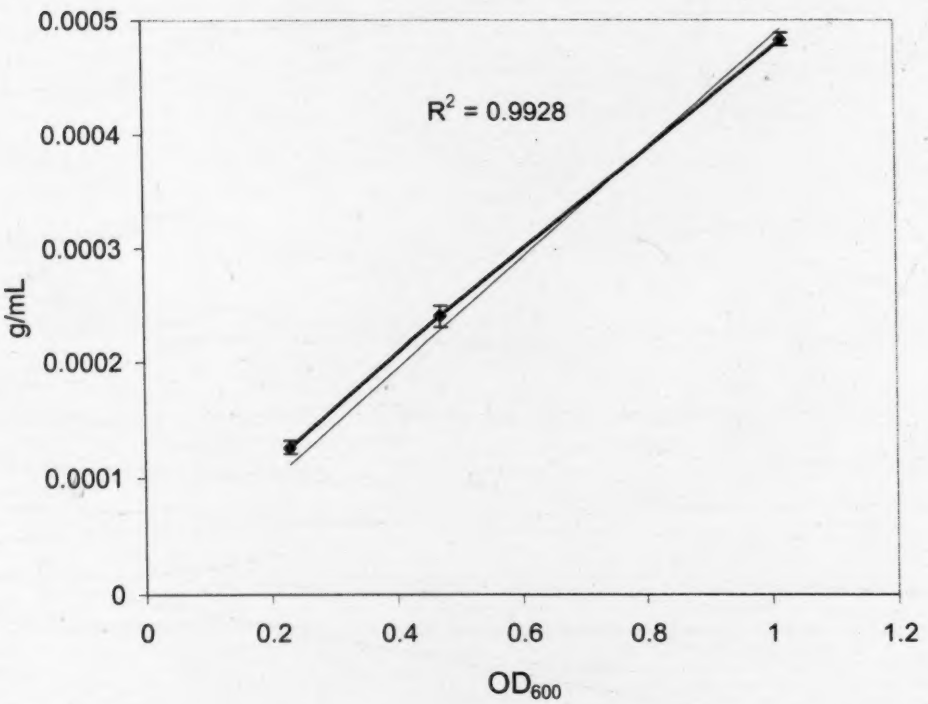

Figure A-6: Dry weight versus OD_{600} for *Rhodococcus erythropolis* 20SE1-c

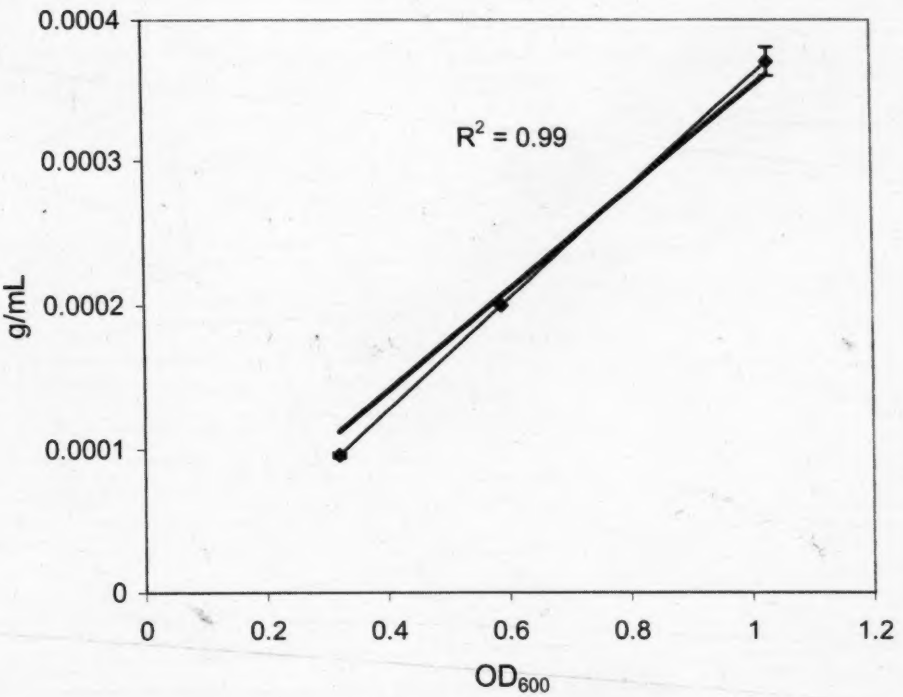

Figure A-7: Dry weight versus OD_{600} for *Pseudomonas fluorescens* LP6a

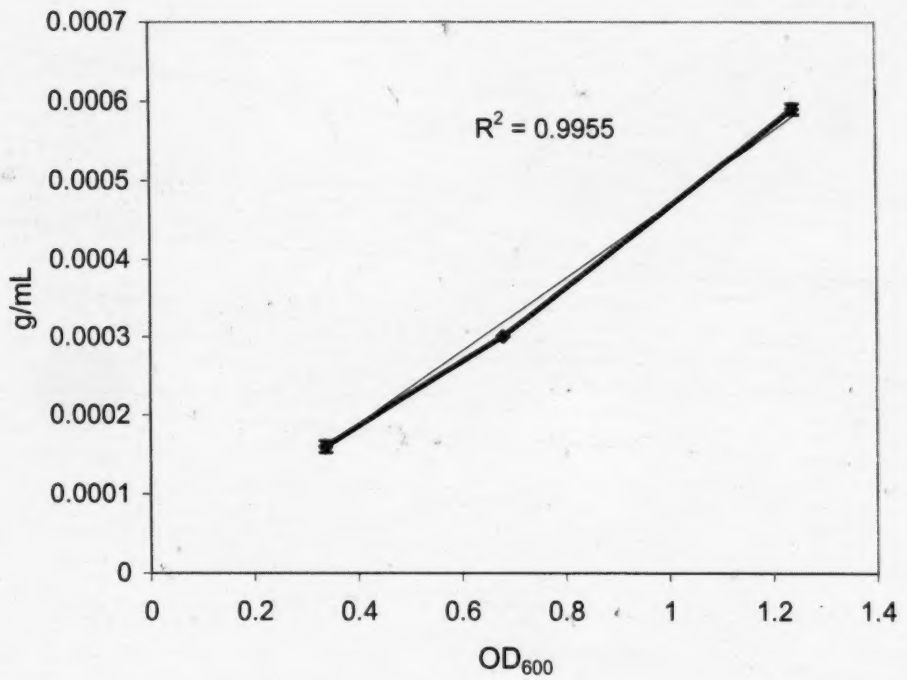

Figure A-8: Dry weight versus OD_{600} for *Rhizomonas suberifaciens* EB2-1a